



Review

Golden rules and pitfalls in selecting optimum conditions for high-speed counter-current chromatography

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Abstract

This paper aims to be an aid to those chemists who are interested in utilizing high-speed counter-current chromatography (HSCCC), which is free of irreversible adsorption and offers high resolution comparable to column chromatography. It explains the selection of HSCCC conditions step by step including the selection of two-phase solvent systems, determination of partition coefficient (K) of analytes, preparation of two-phase solvent system and sample solution, selection of elution mode, flow rate, rotation speed, and on-line monitoring of the eluate. The paper covers both standard HSCCC and pH-zone-refining CCC techniques. Technical terms (*italic*) unfamiliar to the beginner are comprehensively explained in Glossary. Various examples of two-phase solvent systems used in HSCCC are listed in Appendices A and B. The commercial sources of HSCCC and other CCC instruments are described in detail in the study edited by Berthod [A. Berthod (Ed.), Counter-current Chromatography, Elsevier, Amsterdam, 2003].

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Keywords: High-speed counter-current chromatography; pH-zone-refining counter-current chromatography; Type-J multilayer coil planet centrifuge; Partition coefficient; On-line monitoring; Retention of stationary phase

Contents

1. Introduction	146
2. Standard high-speed counter-current chromatography using type-J multilayer coil planet centrifuge	146
2.1. Mechanism	146
2.2. Selection of the two-phase solvent system	148
2.2.1. Partition coefficient	148
2.2.2. Retention of the stationary phase	149
2.3. Sample solution	150
2.4. Separation column	150
2.5. Choice of the mobile phase	151
2.6. Flow rate of the mobile phase	151
2.7. Revolution speed	151
2.8. Filling the column with the stationary phase	151
2.9. Sample loading	152
2.10. On-line monitoring of effluent	152
2.11. Measurement of stationary phase retention	152

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3. pH-zone-refining counter-current chromatography	153
3.1. Mechanism	153
3.2. Selection of the solvent system for pH-zone-refining CCC	154
3.3. Optimization of separation condition	155
3.4. Sample solution	155
3.5. Separation procedure	155
4. Conclusion	156
Acknowledgment	156
References	165
Glossary	166

1. Introduction

In the early 1970s, a new separation technique called *counter-current chromatography*¹ (CCC) was developed. The method provides an advantage over the conventional column chromatography by eliminating the use of a solid support where an amount of stationary phase is limited and dangers of irreversible adsorption from the support are inevitably present. As usual in the development of new methods, the early models had various problems: the first model called helix CCC (now called toroidal coil CCC) [1,2] had a rotary seal and the effluent was introduced from the rotating syringe. This analytical model yielded thousands of *theoretical plates* (TPs), but it required an overnight run. The second model called droplet CCC [2,3] produced a preparative separation at near 1000 TPs, but separating 30 mg of a test sample required 3 days. Unfortunately, the performance of these early models produced a long-standing false image that CCC is a time-consuming technique. In the intervening years, the method has been radically improved in terms of resolution, separation time and sample loading capacity by the development of *high-speed CCC* (HSCCC) [4–7], which yields a highly efficient separation of multigram quantities of samples in several hours. HSCCC, which is one form of CCC, is now accepted as an efficient preparative technique, and widely used for separation and purification of various natural and synthetic products.

The use of this instrumentation, however, requires some simple but special technical knowledge, since the selection of the experimental conditions and the practical separation procedure are quite different from those of conventional column chromatographic methods. During the past 30 years, a number of publications including monographs [8–14], encyclopedia [15,16] and review articles [17] in addition to a great number of research articles on HSCCC in chromatographic journals have been issued. Unfortunately, while exploring these references, I have noticed that these publications are divided into two main categories, one explaining theories, principles and designs, and the other, focused on practical applications. What is lacking, however, are the technical details to be grouped in one place for beginners to follow, i.e.,

how to select a suitable solvent system, how to prepare the sample solution, how to determine the inlet and outlet of the column, how to select the direction and the rotation speed of the apparatus, how to monitor the effluent, etc. Although these procedures are obvious for scientists routinely working on CCC separations, a misuse of one facet may lead to unsuccessful separations and this might well discourage one from continued use of the method.

The purpose of this article is to explain comprehensively the practical CCC procedures for beginners so that they can quickly learn how to use the HSCCC instrument under optimum conditions. To ease understanding the techniques, the mechanisms of both standard HSCCC and *pH-zone-refining CCC* are briefly described with illustrations.

2. Standard high-speed counter-current chromatography using type-J multilayer coil planet centrifuge

2.1. Mechanism

Fig. 1 schematically illustrates the *type-J synchronous planetary motion* of a multilayer coil separation column. The

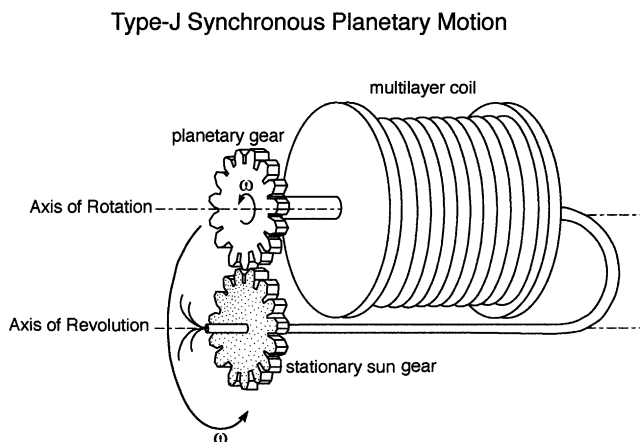


Fig. 1. Type-J planetary motion of a multilayer coil separation column. The column holder rotates about its own axis and revolves around the centrifuge axis at the same angular velocity (ω) in the same direction. This planetary motion prevents twisting the bundle of flow tubes allowing continuous elution through a rotating column without risk of leakage and contamination.

¹ Terms listed in the Glossary are italicised.

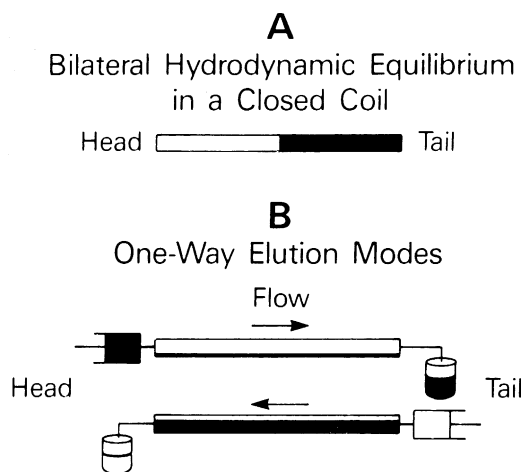


Fig. 2. Mechanism of HSCCC. (A) Bilateral hydrodynamic distribution of two phases in the coiled column. (B) Elution mode of both lighter and heavier phases through the rotating coiled column.

planetary motion is produced by engaging a planetary gear mounted on the column holder axis to an identical stationary sun gear rigidly fixed to the centrifuge framework. This 1:1 gear coupling produces a particular type of planetary motion of the column holder, i.e., the holder rotates about its own axis while revolving around the centrifuge axis at the same angular velocity (synchronous) in the same direction. This planetary motion provides two major functions for performing CCC separation: a rotary-seal-free elution system so that the *mobile phase* is continuously eluted through the rotating separation column. The second and more important function is that it produces a unique hydrodynamic motion of two solvent phases within the rotating multilayer coiled column mainly due to the *Archimedean screw effect*. When two immiscible solvent phases are introduced in an end-closed coiled column, the rotation separates the two phases completely along the length of the tube where the lighter phase occupies one end called the head and the heavier phase, the other end called the tail. (Here, the head and tail relationship is defined according to the Archimedean screw effect: all objects with different densities, either lighter or heavier than the suspending medium, present in the rotating coil are driven toward the head of the coil.) Although the cause of this bilateral hydrodynamic phase distribution of two immiscible solvents is still unknown [18], it can be efficiently utilized for performing CCC as illustrated in Fig. 2.

For simplicity all coils are drawn uncoiled to show the overall distribution of the two phases. In Fig. 2A, the coil at the top shows bilateral hydrodynamic distribution of the two phases in the coil where the white phase (head phase) occupies the head half and the black phase (tail phase) the tail half. This condition clearly indicates that white phase introduced at the tail end will move toward the head and similarly the black phase introduced at the head will move toward the tail. This hydrodynamic trend is effectively used for performing CCC as shown in Fig. 2B. The coil is first entirely filled with the white phase followed by pumping the

black phase from the head end (Fig. 2B, upper diagram). Similarly, the coil is filled with the black phase followed by pumping the white phase from the tail (Fig. 2B, lower diagram). In either case, the mobile phase quickly moves through the coil, leaving a large volume of the other phase stationary in the coil.

The motion and distribution of the two phases in the rotating coil were observed under stroboscopic illumination, and are schematically illustrated in Fig. 3A, where a spiral column undergoes *type-J planetary motion*. (Experimental conditions are described in the figure caption.) The area in the spiral column is divided into two zones: the mixing zone occupying about one quarter of the area near the center of revolution and the settling zone in the rest of the area. In Fig. 3B, the spiral column is stretched and arranged according to the positions I–IV to visualize the motion of the mixing zones along the tubing. It clearly indicates that each mixing zone travels through the spiral column at a rate of one round per

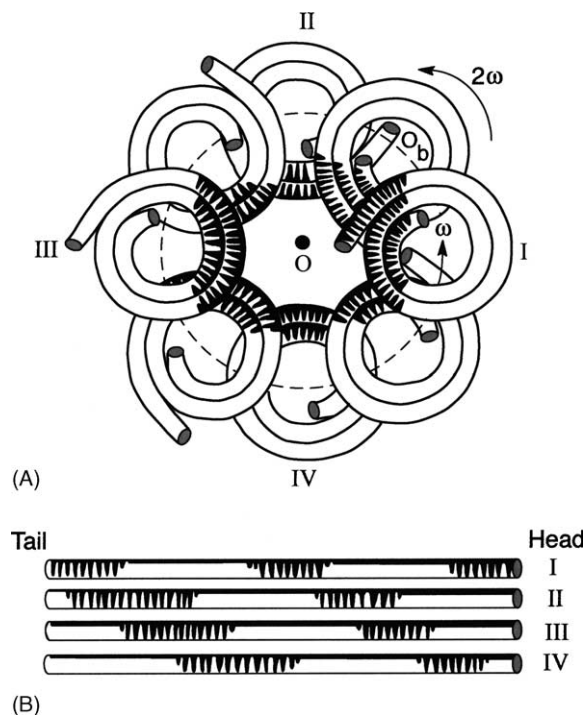


Fig. 3. Schematic drawing of motion and distribution of two phases in the spiral column undergoing type-J planetary motion. Experiment was performed as follows: the spiral column was first completely filled with the aqueous stationary phase followed by rotation of the column at 800 rpm while the red-colored chloroform phase was pumped into the head of the column (inner terminal). Stroboscopic observation was performed after the two phases established steady-state hydrodynamic equilibrium. (A) Whole view of various column positions. The column area is divided into two zones: mixing zone near the center of the centrifuge and settling zone in the rest of the area. (B) Motion of the mixing zones through the stretched spiral column from positions I to IV. It shows that the mixing zone travels through the spiral column at a rate of one round per one revolution of the column. Consequently, it indicates a high partition efficiency of the system that the solutes present at any portion of the column are subjected to a partition process of mixing and settling at an extremely high frequency of 13 times per second at 800 rpm.

one revolution. It indicates the important fact that the solute in the spiral column is subjected to the repetitive partition process of mixing and settling at an enormously high rate of over 13 times per second (at 800 rpm). It demonstrates the high efficiency of HSCCC.

2.2. Selection of the two-phase solvent system

Conventional liquid chromatography uses a single phase to elute the analytes released from the adsorptive or liquid phase coated solid support. In contrast, the CCC technique uses a *two-phase solvent system* made of a pair of mutually immiscible solvents, one used as the *stationary phase* and the other as the *mobile phase*. The use of two-phase solvent systems allows one to choose solvents from an enormous number of possible combinations. The selection of this two-phase solvent system for the target compound(s) is the most important step in HSCCC where searching for a suitable two-phase solvent system may be estimated as 90% of the entire work in HSCCC. Without consulting the literature, the search for a two-phase solvent system for the successful separation of particular compounds from a complex sample mixture can be very time-consuming. Therefore, **Rule 1: First study previous articles on the CCC or CCD (counter-current distribution) (see Appendix) involving separation of similar compounds.** Several monographs, review articles, and book chapters in the cited references describe various two-phase solvent systems successfully used for CCC. Refs. [11,14,17] provide extensive lists of two-phase solvent systems for HSCCC, while **Appendices A and B** list the recommended representative examples. If none of these are successful in their initial or modified form, then one faces the challenge of inventing new conditions. The solvent systems used for *counter-current distribution* can also be equally well applied to HSCCC [19,20].

When the search for a solvent system is unsuccessful, one must resort to a tedious trial and error method to find a suitable two-phase solvent system. Those who have worked on natural products purification use empirical methods for relatively efficiently finding a suitable system [21,22]. The selected solvent system should satisfy the following requirements: (1) the analyte(s) should be stable and soluble in the system; (2) the solvent system should form two phases with an acceptable volume ratios to avoid wastage; (3) the solvent system should provide a suitable *partition coefficient* to the analytes; and (4) the solvent system should yield satisfactory *retention of the stationary phase* in the column. Since the first two requirements are obvious, we consider below the partition of analytes and retention of the stationary phase for HSCCC separation.

2.2.1. Partition coefficient

The *partition coefficient* (K) is the ratio of solute distributed between the mutually equilibrated two solvent phases. Usually it is expressed by the amount of solute in the stationary phase divided by that of the mobile phase as in conventional liquid chromatography. **Rule 2: Find sys-**

tems with K values of the target compounds in a proper range: The suitable K values for HSCCC are $0.5 \leq K \leq 1.0$.

A smaller K value elutes the solute closer to the solvent front with lower resolution while a larger K value tends to give better resolution but broader, more dilute peaks due to a longer elution time. In CCC, one can choose either upper or lower phase as the stationary phase (depending on head-to-tail versus tail-to-head elution as discussed later). Before deciding which phase is to be used as the stationary phase, the user therefore may temporarily express the partition coefficient as $K_{U/L} = C_U/C_L$, where C_U is the solute concentration in the upper phase and C_L , that of the lower phase. If $K_{U/L} = 2$, the lower phase should be used as the stationary phase, which gives $K = 0.5$. It is important that this preliminary $K_{U/L}$ must be clearly distinguished from K using the subscripts to avoid confusion.

The measurement of the $K_{U/L}$ values may be performed as follows: Add a small amount (typically a few milligrams or less depending on its extinction coefficient or absorptivity) of each target compound (one at a time unless quantified later in the two phases by HPLC) to the two mutually equilibrated solvent phases (1–2 ml each) in a stoppered test tube (13 mm × 100 mm). Thoroughly mix with a vortex to equilibrate the contents. After settling, pipette and deliver an equal volume of the upper and the lower phases (100–200 μ l) each into a separate test tube, dilute each with an equal volume (2 ml) of a suitable solvent such as methanol, and measure the absorbance by a spectrophotometer at the suitable wavelength to obtain the $K_{U/L}$ value. If the pure sample is not available, one can subject each phase to HPLC analysis to compare the peak height (or area under the peak) of the corresponding peaks. If the sample does not absorb in the UV or at a visible wavelength, the $K_{U/L}$ value may be determined by various other ways such as TLC (color reaction), evaporative light scattering detector (ELSD), refractive index, MS, dry mass and biological activity. TLC combined with densitometry is effectively used for simultaneous determination of $K_{U/L}$ of multiple components from a sample mixture.

Following the procedure described above for the measurement of $K_{U/L}$, see **Rule 3: Search systematically for a suitable solvent system according to Tables 1 and 2 [14,15,23]**, in which sets of two-phase solvent systems are arranged from top to bottom in decreasing order of hydrophobicity in the organic phase. When the polarity of the target compounds is unknown, the search may start with the two-phase solvent system composed of hexane–ethyl acetate–methanol–water at a volume ratio of 3:5:3:5 (Table 1) which has a moderate degree of polarity. If the partition coefficient is slightly off from the proper range, it can be adjusted by modifying the volume ratio. (For example, if $K_{U/L}$ is slightly over 2, the volume ratio may be modified toward more hydrophobic such as 3.2:5:3.2:5, and if $K_{U/L}$ is slightly less than 0.5, the volume ratio may be modified in the opposite direction to 2.8:5:2.8:5.) If the target compound is mostly distributed in the upper organic phase, the search is directed upward along the arrow. If it is mostly distributed in the lower aqueous phase, the search

Table 1
Search for the suitable two-phase solvent system

	n-Hexane	EtOAc	MeOH	n-BuOH	water	
	10	0	5	0	5	↑ HYDROPHOBIC
	9	1	5	0	5	
	8	2	5	0	5	
	7	3	5	0	5	
	6	4	5	0	5	
	5	5	5	0	5	
Start ↑ here ↓	4	5	4	0	5	
	3	5	3	0	5	
	2	5	2	0	5	
	1	5	1	0	5	
	0	5	0	0	5	
	0	4	0	1	5	POLAR ↓
	0	3	0	2	5	
	0	2	0	3	5	
	0	1	0	4	5	
	0	0	0	5	5	

Systematic search for suitable solvent systems for HSCCC. A set of solvent systems is arranged from the top to the bottom according to a decreasing order of the hydrophobicity of their organic phases. When the hydrophobicity of the target analyte is unknown, the search may start at hexane–ethyl acetate–methanol–water (3:5:3:5, v/v) and then follow the direction indicated by a pair of arrows. Importantly, each of these solvent systems gives nearly a 1:1 phase volume ratio, conserving the solvent in CCC and making it easier to separate the two phases both during evaluation in the test tube, and when preparing phases in a separatory funnel.

should be directed downward along the arrow or apply the solvent system described at the top of Table 2. The search should be continued until a suitable range of $K_{U/L}$ values for all of the compounds of interest is obtained. When the search reaches the solvent system at the upper end of Table 1 and the analyte(s) is still distributed more into the organic upper phase, one may test extremely hydrophobic solvent systems

Table 2
Series of polar MBE solvent systems

MBE–1-BuOH–ACN–water
1:0:0:1
4:0:1:5
6:0:3:8
2:0:2:3
6:4:5:5
2:2:1:5

MBE: methyl *tert*-butyl ether; BuOH: butanol; ACN: acetonitrile. Systematic search for suitable solvent systems for pH-zone-refining CCC. A set of solvent systems is arranged from the top to the bottom according to a decreasing order of the hydrophobicity of their upper organic phases. The search may start at the top of the table. As in the solvent systems listed in Table 1, each of these solvent systems gives nearly a 1:1 phase volume ratio, conserving the solvent in CCC and making it easier to separate the two phases both during evaluation in the test tube, and when preparing phases in a separatory funnel.

such as hexane–ethanol–water (5:4:1) or hexane–methanol. If the search reaches the lower end of Table 1, where the analyte distributes more to the aqueous phase in 1-butanol–water, one may add a modifier to the systems: e.g., 1-butanol–acetic acid–water (4:1:5); 1-butanol–0.1% aqueous trifluoroacetic acid (TFA) (1:1); and 1-butanol–0.2 M NaCl (1:1). The solvent systems illustrated in Table 2 are extremely useful for pH-zone-refining CCC described later.

If the sample is an extract of plant material, the search may start at any point according to the polarity of the solvent used for the extraction: if the sample is an ethyl acetate extract (relatively hydrophobic solvent), the search may start at hexane–ethyl acetate–methanol–water (1:1:1:1), whereas if the sample is a methanol extract (polar solvent), the search may start at 1-butanol–water.

When using neutral solvent systems listed in Tables 1 and 2, consider **Rule 4: Modify the K value of the negatively-charged analytes, e.g., carboxylic acids, by adding acids such as TFA and acetic acid to the solvent system.** Due to protonation, these molecules become more hydrophobic and favor partition to the organic phase. Since these acidic analytes have two molecular forms, protonated ($-\text{COOH}$) and deprotonated ($-\text{COO}^-$), each having a different K value, they form a broader peak when partly ionized. Therefore, the use of an acidic modifier to the solvent system is recommended. Also, adding the acid (typically 0.1% TFA) to the solvent system often substantially shortens the *settling time*, improving retention of the stationary phase. This is especially true when the sample solution contains acidic impurities.

The K values provide useful information on resolution between two similar compounds; see **Rule 5: The ratio of the two K values or the separation factor ($\alpha = K_1/K_2$, where $K_1 > K_2$) (Glossary) ought to be greater than 1.5 in the semipreparative multilayer separation column of a commercial HSCCC unit.** Also, from the partition coefficient value of the analytes, one can predict the retention volume needed to elute the analyte according to **Rule 6: If K is 1, the analyte will elute at the retention volume equal to the column capacity regardless of the retention volume of the stationary phase. At $K < 1$ or $K > 1$, the analyte would elute before or after the elution of one column capacity volume, respectively.** The retention volume (V_R) of the analyte may be predicted more accurately using the elution volume of the solvent front (V_{SF}) and the total column capacity (V_C) by the following equation:

$$V_R = V_{SF} + K(V_C - V_{SF}) \quad (1)$$

2.2.2. Retention of the stationary phase

The CCC system uses no solid support to retain the liquid stationary phase in the separation column. In HSCCC, the retention of the stationary phase is accomplished by a combination of coiled column configuration and the planetary motion of the column holder. Successful separation in HSCCC largely depends on the amount of the stationary phase retained in the column. In general, **Rule 7: The higher the**

Table 3
Preparation of sample solution for standard HSCCC and pH-zone-refining CCC

	Standard HSCCC	pH-zone-refining CCC
Sample volume	Preferably less than 5% of column volume	Can be a large volume as far as it is retained in the column
Solvent	<i>Mobile phase:</i> good if $K > 0.5$. <i>Stationary phase:</i> good for $K \leq 0.5$. (In each case add a small amount of the other phase until two phases formed.) <i>Both phases:</i> minimizes the sample volume when the sample contains solutes with a broad range of polarity. This may be the best choice	Use both phases: the stationary phase with retainer (and counterions such as DEHPA, if needed) and mobile phase free of eluter. Check K values to see the most of solutes are in the stationary phase ($K > 5$). If not, add more retainer to increase the K value. Measure the settling time (t s), and if $t > 20$ s, dilute the sample solution to adjust to $t < 20$ s
Precipitate	Remove any solid matter by centrifugation or filtration	Undissolved matter can be charged after sonication

retention of the stationary phase, the better the peak resolution.

The amount of stationary phase retained in the column is highly correlated with the *settling time* (Glossary) of the two phases in a test tube [24]. **Rule 8: Measure the settling time of the two-phase solvent system to be used for the separation.** The procedure is as follows: the two phases are first equilibrated in a separatory funnel. Deliver 2 ml of each phase, a total volume of 4 ml, into a test tube (13 mm × 100 mm) or a graduated cylinder (5 ml capacity), which is then capped. Gently invert the container for several times and then immediately place it in an up-right position to measure the time required for the two phases to form clear layers with a distinct interface. If the settling time is less than 20 s, the solvent system would provide satisfactory retention of the stationary phase, usually over 50% of the total column capacity, in a proper range of flow rates.

All the series of two-phase solvent systems listed in Tables 1 and 2 provide satisfactory retention of the stationary phase and yield about equal volumes of each phase. Although retention of over 50% is ideal, a less amount, such as 30%, may still give a satisfactory separation, especially for the analytes with high K values.

2.3. Sample solution

Preparation of the sample solution for HSCCC needs some consideration (Table 3). The sample may be dissolved directly in the stationary phase or in a mixture of the two phases. The recommended sample volume in the standard separation using the semipreparative column of the commercial HSCCC unit with *partition efficiency* of about 600–800 TPs may be less than 5% of the total column capacity (this is a rough measure based on an analogy with the counter-current distribution method). Introduction of a larger sample volume into the column will reduce peak resolution of the analytes especially with those having small K values. Ideally the analyte is injected in a small volume of the stationary phase to preserve the sharpness of the early elution peak, since the mobile phase then further concentrates the analyte in the sample compartment in the column. When a sample contains solutes with a broad range of hydrophobicity, one can minimize the necessary sample volume by dissolving it into a mixture of two phases (by dissolving hydrophobic components into the organic phase and polar components into the aqueous phase).

When the sample is not easily dissolved in either phase even after sonication, the sample may be initially dissolved in the solvent in which the analyte has the highest solubility. For example, if the sample is an ethyl acetate extract and the selected two-phase system is hexane–ethyl acetate–methanol–water, the sample may be first completely dissolved in ethyl acetate (extraction solvent), followed by gradually adding methanol, hexane and water in this order according to their volume ratio. If a precipitate is formed during this process, one may either add more solvent (upper and/or lower phases) until the precipitates are completely dissolved or eliminate the precipitates by centrifugation. If the K value of the target compound in the solvent system is selected in a proper range, such precipitates are most likely to be impurities. (However, it is recommended that one should keep these precipitates for later analysis.)

Rule 9: Ensure that the sample solution forms two phases when mixed with either phase in the column. When the sample is dissolved only in the stationary phase, a small amount of the mobile phase should be added until the solution becomes cloudy, indicating formation of two phases. When a large amount of sample is dissolved in a two-phase mixture, the sample solution often forms a single phase due to the altered phase composition. When this occurs, more solvent (the upper and/or the lower phases) should be added until a distinct interface is formed between the two phases. *If this is not followed, the mobile phase might push the stationary phase almost entirely from the column.* In order to ensure satisfactory retention of the stationary phase, dissolve the sample in about equal volumes of the two phases and measure the settling time of the sample solution using the method described earlier. If the settling time becomes much longer than 20 s, the sample solution should be further diluted until the settling time is within an acceptable range even though the sample volume exceeds 5% limit of the total column volume.

2.4. Separation column

HSCCC uses a multilayer coil separation column, which is prepared by winding a long piece of PTFE tubing directly around the spool-shaped column holder making multiple layers. As mentioned previously, when this coiled column is subjected to a planetary motion, it produces a familiar effect called “Archimedean screw” which drives all objects of

different density, either lighter or heavier than the suspended medium, toward one end of the coil called the “head”; the other end is called the “tail” (see *Archimedean screw effect* in Glossary). In order to properly operate the HSCCC instrument (*type-J planetary motion*), the user must understand the head–tail orientation of the separation column: **Rule 10: A lower (heavier) mobile phase should be introduced through the head toward the tail, and an upper (lighter) mobile phase in the opposite direction. This is extremely important because the elution of either phase in the wrong direction results in an almost complete loss of the stationary phase from the column.** Since the head and tail relationship is switched by changing the direction of column rotation, each flow tube, inlet or outlet, should be defined in the following way: Head when clockwise revolution (CW) and Tail when counter-clockwise revolution (CCW). The head end of the column is easily determined by introducing a small volume (5–10 ml) of water into an empty column and rotating the column at a low speed (200–300 rpm) while keeping the ends of both flow tubes in a reservoir of water. Air bubbles will soon appear from the flow tube connected to the head of the column.

The separation column (multilayer coil) of the commercial HSCCC instrument is usually made of the following three sizes of PTFE tubing: 2.6 mm i.d. (preparative), 1.6 mm i.d. (semipreparative) and 0.85–1.0 mm i.d. (analytical). The analytical column is useful for optimization of the solvent system, since the same solvent system can be used to obtain the same elution profile in a large scale.

2.5. Choice of the mobile phase

In HSCCC, either phase can be used as the mobile phase provided that the K value of the analyte is in a proper range (Table 4). If one has a choice, the lower phase may be used as the mobile phase, because the system provides more stable retention of the stationary phase (less carryover and better tracing of the elution peaks) and one can avoid trapping air bubbles in the flow cell of the detector by introducing the effluent from the lower end of the cell (see below). On the other hand, when the upper organic mobile phase (excluding the chloroform system) is used as the mobile phase, it will facilitate the evaporation of solvent from the collected fractions.

Table 4
Choice of the mobile phase

	Upper organic phase mobile	Lower aqueous phase mobile
Elution mode ^a	Tail to head ($t < 20$ s)	Head to tail ($t < 20$ s)
K values ^b	$1.0 \leq K_{U/L} \leq 2.0$	$0.5 \leq K_{U/L} \leq 1.0$
Retention of stationary phase	Depending on t values: when $t > 20$ s, retention may be unstable associated with carryover of the stationary phase	Usually stable and minimum carryover
On-line UV-detection	Possible bubble trapping in the flow cell to disrupt the tracing of elution curve	No air bubble problem and produces the tracing of elution curve with minimum noise
Drying fractions	Easy to dry fractions	Takes a longer time

^a t : settling time (Glossary).

^b $K_{U/L}$: solute concentration in the upper phase divided by that in the stationary phase.

2.6. Flow rate of the mobile phase

The flow rate of the mobile phase determines the separation time, the amount of stationary phase retained in the column, and therefore the *peak resolution*. A lower flow rate usually gives higher retention level of the stationary phase [25] improving the peak resolution although it requires a longer separation time. The typical flow rates for the commercial multilayer coil are as follows: 5–6 ml/min for a preparative column with 2.6 mm i.d. PTFE tubing (600–800 rpm) (up to 1 g sample load); 2–3 ml/min for a semipreparative column with 1.6 mm i.d. PTFE tubing (800–1000 rpm) (up to 500 mg sample load); and 1 ml/min for an analytical column with 0.85–1.0 mm i.d. PTFE tubing (1000–1200 rpm) (up to 50 mg sample load). The above range of flow rates should be modified according to the *settling time* of the two-phase solvent system as well as other factors. When the settling time is around 20 s and the K value of the analyte is small, the use of a lower flow rate is recommended.

2.7. Revolution speed

The optimum revolution speed (revolution and planetary rotation speeds are always same) for the commercial HSCCC instrument for preparative separation ranges between 600 and 1200 rpm ($40\text{--}160 \times g$) with a 10 cm revolution radius according to the i.d. of the separation coil as described above. Use of a lower speed will reduce the volume of the stationary phase retained in the column leading to lower peak resolution. On the other hand, the higher speeds may produce excessive sample band broadening by violent pulsation of the column because of elevated pressure.

2.8. Filling the column with the stationary phase

In each separation, the column is first entirely filled with the stationary phase. It is recommended that before introducing the stationary phase the column may be flushed with a column volume of a solvent miscible with the two phases used in the previous run (e.g., ethanol or methanol) to wash out materials still remaining in the column. This will also ensure a stable clean baseline before the solvent front emerges.

Rule 11: Avoid trapping the air in the column especially in a preparative column. This can be tested as follows: If

no air is present in the column, the flow from the column outlet is ceased shortly after stopping the pumping. If the solvent keeps flowing out from the outlet for over several seconds, the air trapped in the column should be eliminated by resuming the pumping of the stationary phase under low speed column rotation (100–200 rpm) in a tail to head elution mode to accelerate air movement toward the outlet of the column.

2.9. Sample loading

There are two ways to carry out the procedure. Both are satisfactory for the HSCCC separation. In the first method, the column is first entirely filled with the stationary phase and this is immediately followed by sample injection. The mobile phase is then eluted through the column while the column is rotated at the optimum rate. In the second method, after the column is filled with the stationary phase, the mobile phase is eluted through the column at a desired rate until the solvent front emerges and *hydrodynamic equilibrium* is established throughout the column as evidenced by diminished *carryover of the stationary phase*. The sample is then injected into the column through the sample port. Each method has its own advantages. It is obvious that the second method will give a clear tracing of the elution curve because of the minimum carryover of the stationary phase from the column. The first method produces a distinct solvent front and saves separation time by eliminating the waiting period to reach hydrodynamic equilibrium.

One can conveniently use an injection valve with a sample loop as in HPLC. The method allows successive analysis of multiple samples at suitable intervals without renewing the column contents. This is particularly useful for determination of *log P* values of various drugs.

2.10. On-line monitoring of effluent

The effluent from the outlet of the HSCCC column may be continuously monitored by a UV–vis detector as in conventional liquid chromatography. An important difference between these two methods is that HSCCC uses the liquid stationary phase, which if carried over from the column, tends to disturb the tracing of the elution curve. **Rule 12: Avoid trapping the stationary phase in the vertical flow cell by eluting the lower mobile phase upward from the bottom and the reverse if the upper is used as the mobile phase.**

When the upper mobile phase is eluted from the top of the flow cell downward, it is important to prevent formation of air bubbles which might be trapped in the flow cell and disturb the tracing of the elution curve. This problem can be largely avoided by *degassing* the two phases in the separatory funnel before use, and also by connecting fine PTFE tubing (typically 30 cm × 0.4–0.5 mm i.d.) to the outlet of the monitor so that the pressure within the flow cell is substantially increased to prevent bubble formation. The tracing problem by carryover of the stationary phase is avoided using evapo-

rative light scattering detection (ELSD) if the mother solvent contains no solid component.

For analytes without chromophores, on-line monitoring of the effluent can be performed by various ways, e.g., using ELSD [26], mass spectrometry (HSCCC–MS) [27] or IR [28]. Although on-line is recommended, it is not necessary if one has optimized the solvent system so that the target compound elutes after a reasonable volume predicated by its *K* value. After collecting fractions, one can evaluate them by HPLC or TLC combined with densitometry, although on some occasions it may be necessary to drying the fractions and redissolving the residues in an appropriate solvent.

2.11. Measurement of stationary phase retention

When the separation is completed, rotation is stopped and the column contents are collected into a graduated cylinder by connecting the column inlet to a nitrogen cylinder (ca. 50 p.s.i.; 1 p.s.i. = 6894.76 Pa). After nitrogen appears at the outlet, the column is slowly rotated (100 rpm) in the tail to head *elution mode* (Glossary) so that solvent remaining inside the column is pumped out by an Archimedean screw force assisted by the nitrogen flow. Instead, one may stop the rotation and continuously elute the column with the mobile phase at a higher rate to collect the retained stationary phase in a graduated cylinder. Here again, slow column rotation in the tail to head mode will help the recovery of stationary phase from the column. From the volume of the stationary phase recovered, the % retention of the stationary phase is estimated by dividing it by the total volume collected, or dividing by the total column capacity assuming that the amount of solvent still remaining in the column is negligible. Alternatively, retention of the stationary phase may be estimated from the retention volume of the solvent front, provided that carryover of the stationary phase is minimum.

The retained stationary phase volume (V_S) may also be computed from the chromatogram using the retention volume of the peak (R) and the *K* value(s) according to the following two methods:

$$V_S = \frac{R - V_{SF}}{K} \quad (2)$$

where V_{SF} is the solvent front, or alternatively

$$V_S = \frac{R_2 - R_1}{K_2 - K_1} \quad (3)$$

where R_2 and R_1 give the retention volume of peaks 2 and 1; and K_2 and K_1 provide the *K* values of peaks 2 and 1, respectively.

Measurement of the stationary phase retention is useful in efforts to improve the separation: when the peak resolution is unsatisfactory, a measure of the stationary phase retention will serve as a guide for the next trial. If it is less than 30%, the separation may be improved by increasing retention by applying a lower flow rate of the mobile phase, increasing the revolution speed, or modifying the solvent system to

shorten the *settling time*. If instead the stationary phase retention is over 50%, efforts should be directed to search for a new two-phase solvent system, which provides an improved *separation factor* (α) between the analytes.

3. pH-zone-refining counter-current chromatography

pH-zone-refining CCC [29–33] is generally employed as a large-scale preparative technique for separating ionizable analytes. The method elutes highly concentrated rectangular peaks fused together with minimum overlapping while impurities are concentrated and eluted between the outside the major peaks according to their pK_a and hydrophobicity. Although it somewhat resembles displacement chromatography, there are distinct differences between these two methods [30].

The greatest advantage of the method is its large sample loading capacity, which exceeds 10-fold that of the standard HSCCC in the same separation column. In addition, the method provides various special features such as yielding highly concentrated fractions, concentrating minor impurities for detection, and allowing the separation to be monitored by the pH of the effluent when there are no chromophores.

Selection of solvent systems and preparation of the sample are quite different from those used in the standard HSCCC technique.

3.1. Mechanism

This preparative CCC technique was originated from an incidental finding that the acidic solute (bromoacetyl- T_3) formed an extremely sharp peak if the sample solution contained a strong acid (bromoacetic acid) [34]. The mechanism of this peak sharpening is shown in Fig. 4 where a portion of the separation column contains the organic stationary phase in the upper half and the aqueous mobile phase in the lower half

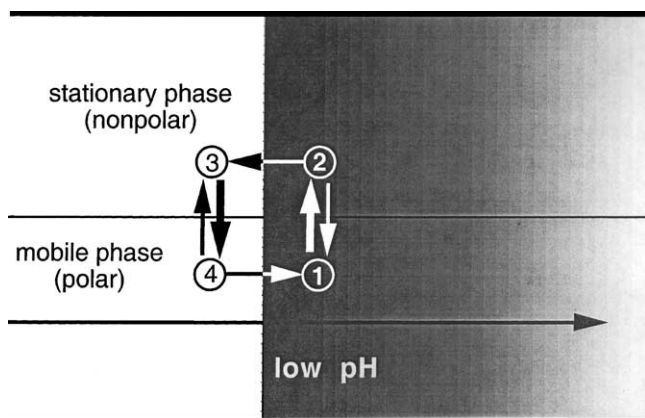
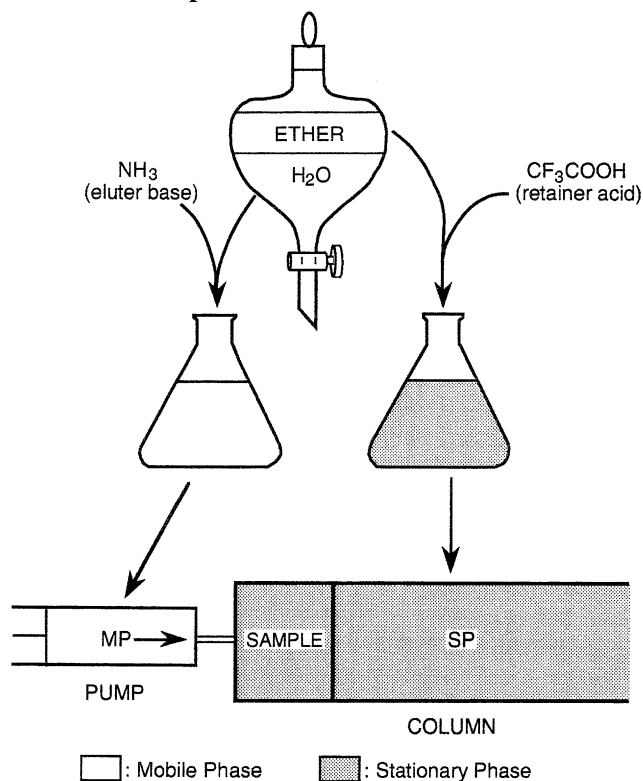


Fig. 4. Mechanism of sharp peak formation. The acid analyte is always confined around the sharp retainer border and elutes as a sharp peak with the retainer acid.

as indicated. Due to its *non-linear isotherm* the acid present in the sample solution forms a sharp trailing border, which travels through the column at a rate lower than that of the mobile phase. As indicated in the diagram, the right side of the sharp border gives much lower pH than the left side. Under this condition the acid analyte present in position 1 is protonated and quickly transferred into the stationary phase at position 2. As the sharp acid border moves forward, the analyte is exposed to high pH, deprotonated, and moves into the mobile phase at position 4. Then, the mobile phase quickly carries the analyte, which passes the sharp acid border to repeat the above process. Consequently, the analyte is always confined near the sharp acid border and finally elutes as a sharp peak with the sharp acid border. This phenomenon is more effectively reproduced by introducing an acid (such as TFA) (*retainer*) in the stationary phase and a base (such as NH_4OH) (*eluter*) in the mobile phase. Further, it has been observed that the sharp peak becomes a rectangular peak when the sample size is increased. This may be explained as follows: when the amount of acid analyte is accumulated behind the trailing acid sharp border, pH is locally reduced resulting in formation of the second sharp peak trailing border, which eliminates the other acidic compounds with higher pK_a and hydrophobicity. The same process takes place again for the third acid analyte with still higher pK_a and hydrophobicity and so forth for the remaining acid analytes. This process is demonstrated in the following model experiment illustrated in Fig. 5.

In Fig. 5A, a two-phase solvent system composed of ether and water is equilibrated in a separatory funnel and separated. TFA (retainer) is added to the upper organic phase, and NH_4OH (eluter) to the lower aqueous phase. The column is first completely filled with the acidified organic stationary phase followed by the injection of sample solution containing three acidic analytes, S_1 , S_2 and S_3 . Then, the basified aqueous phase is pumped into the column while the column is rotated at an optimum speed. Fig. 5B (upper diagram) illustrates a portion of the separation column which shows the stationary phase in the upper half and the mobile phase in the lower half. The retainer acid, TFA, forms a sharp trailing border (due to its non-linear isotherm), which moves through the column at a rate lower than that of the mobile phase. Three analytes, S_1 , S_2 and S_3 , competitively form solute zones behind the sharp TFA border according to their pK_a and hydrophobicity. Among these, S_1 , with the lowest pK_a and hydrophobicity is located immediately behind the TFA border, while S_3 , with the highest pK_a and hydrophobicity is located at the end of the solute zones where it forms a sharp trailing border. As indicated by curved arrows, proton transfer takes place at each zone boundary governed by the difference in pH between the neighboring zones. The loss of the solute from the mobile phase to the stationary phase is compensated by its return at the back of each zone while the ammonium ion in the aqueous phase serves as a counterion for all species. After equilibrium is reached, the three solute zones move at the same rate as that of the TFA border, while

A. Preparation for model experiment



B. Mechanism of pH-zone-refining CCC

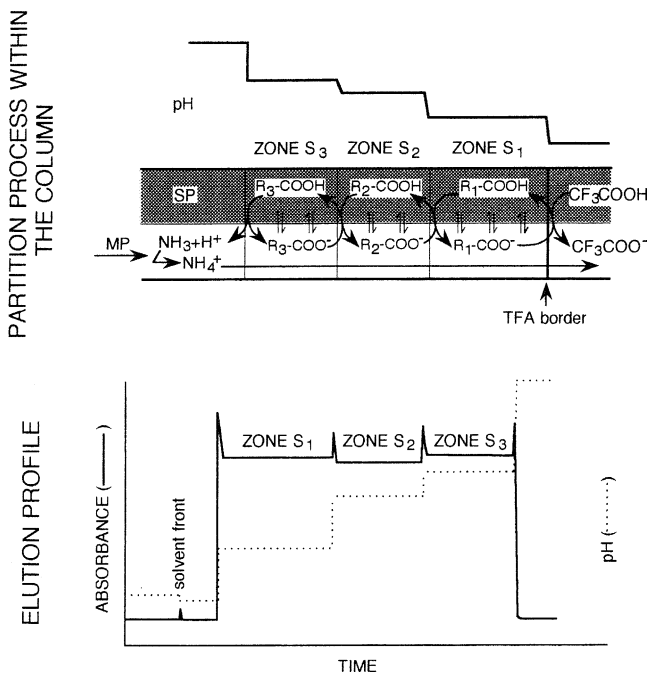


Fig. 5. Model experiment to demonstrate the mechanism of pH-zone-refining CCC. (A) Preparation of the mobile and stationary phases for separation of acid analytes. (B) pH-zone formation of three analytes in the column (upper diagram), and the elution profile of the analytes (lower diagram).

constantly maintaining their width and pH. Charged minor components present in each zone are efficiently eliminated either forward or backward according to their partition coefficients (pK_a and hydrophobicity) and eventually accumulate at the zone boundaries. Consequently, the three analytes elute as a train of rectangular peaks with sharp impurity peaks at their narrow boundaries as illustrated in Fig. 5B (lower diagram).

3.2. Selection of the solvent system for pH-zone-refining CCC

Solvent systems, which have been successfully used for pH-zone-refining CCC, are described in several monographs and review articles [30–32] (see Appendix B). Since the analytes are ionizable compounds, most separations can be performed using relatively polar solvent systems listed in Table 2. Unless the analytes are hydrophobic compounds such as long-chain fatty acids, one can start at a two-phase solvent system composed of methyl *tert*-butyl ether–water by following step by step the partition procedures indicated below [30]: **Rule 13: For an acidic analyte follow these steps:**

- (1) A 2 ml volume of each phase and 5 μ l of NH₄OH (ca. 28% NH₃ stock solution) (eluter) is delivered into a test tube (13 mm \times 100 mm) or bring the pH above 10.
- (2) Add a small amount of the analyte (so that no significant change is made in pH), apply a stopper and vortex several times to equilibrate the contents.
- (3) Measure the analyte concentration in the upper and the lower phases to obtain $K_{U/L}$ value or K_{base} .
- (4) If $K_{base} \ll 1$, add TFA (retainer) (ca. 20 mM) to the contents to bring the pH to around 2, and reequilibrate the contents by vortexing.
- (5) Using the procedure in Step (3), obtain K_{acid} , and if $K_{acid} \gg 1$, the solvent composition is suitable for separation.
- (6) If K_{base} is not small enough, repeat the whole procedure using a less polar solvent system such as hexane–ethyl acetate–methanol–water, 1:1:1:1 in Table 1 and move upward.
- (7) If K_{acid} is not large enough, repeat the whole procedure using a more polar solvent system of methyl *tert*-butyl ether (MBE)–acetonitrile (ACN)–water (2:2:3) in Table 2 and downward.

Rule 14: For a basic analyte, substitute HCl for NH₄OH at Step (1) to test $K_{acid} \ll 1$, and substitute triethylamine for TFA at Step (4) to test $K_{base} \gg 1$.

For zwitterion (free peptides, etc.) or highly polar analytes (catecholamines), the use of a hydrophobic counterion is required to retain the analyte in the stationary organic phase. A successful separation may be performed using di(2-ethylhexyl)phosphoric acid (DEHPA) for peptides and catecholamines [30], and dodecylamine for sulfonated dyes [33]. For *chiral separations* [35,36], a suitable chiral selector

such as *N*-dodecanoyl-L-proline-3,5-dimethylanilide is dissolved in the stationary phase: the higher its concentration, the greater the resolution.

All of these selectors are dissolved in the stationary phase at the optimum concentration determined by the preliminary study. One important requirement is that the selector must be almost entirely distributed in the stationary phase. The use of a selector such as DEHPA requires that the solvent pH be kept at neutral or in an acidic range in the column by eluting with a proper concentration of HCl in the mobile phase to prevent intensive emulsification of the two solvent phases.

3.3. Optimization of separation condition

After the solvent composition is determined, the retainer (typical concentration of 10–20 mM) is added to the organic phase and the eluter (about equal molar concentration to the retainer) is added to the aqueous phase. The concentration of eluter in the mobile phase mainly determines the concentration of analyte in the eluted fractions. Therefore, increasing the eluter concentration results in a higher concentration and shorter retention time of the analyte. On the other hand, the concentration of the retainer in the stationary phase determines the concentration of analyte in the stationary phase within the column. Consequently, increasing the retainer concentration raises the concentration of analytes in the stationary phase associated with a longer retention time or increased *K* value of the analyte.

In general, a large amount of sample can be effectively separated by applying high concentrations of both retainer and eluter, e.g., 40 mM each. However, use of such high retainer concentration often induces carryover of the stationary phase, apparently caused by precipitation of the analyte due to its excessively high concentration or limited solubility in the stationary phase. **Rule 15: Start with equal molar concentrations of retainer and eluter such as 10–20 mM each.**

The method allows the use of an organic phase as the mobile phase (in this case, the retainer becomes the eluter and vice versa) [30]. In this operation, the retainer in the aqueous stationary phase serves as the counterion for the retained analytes to determine their concentration in the stationary phase. The eluter in the organic phase, on the other hand, modifies the partition coefficient of analytes in such a way that increasing the eluter concentration shortens the retention time and increases the concentration of analyte in the mobile organic phase. In this mode, 10–20 mM each of the eluter and retainer may also produce a satisfactory result.

3.4. Sample solution

The preparation of sample solution for pH-zone-refining CCC is different from that used in the standard HSCCC separation. A typical sample size to be separated by a commercial semipreparative HSCCC unit with a 320 ml column capacity may range from 0.5 to 5 g depending on the solubility of the

sample in the solvent system (which is about 10 times that used for the standard HSCCC separation). The sample solution is prepared by dissolving a desired amount of sample in the stationary phase containing the retainer (and hydrophobic counterion or chiral selector) and adding a lesser amount of the mobile phase free of eluter. Although it is ideal to completely dissolve the sample, a sample solution containing undissolved solute may be introduced into the column after making a fine homogeneous suspension by sonicating for several minutes. Alternatively, the precipitates may be removed by centrifugation for later analysis. In pH-zone-refining CCC, the analyte concentration in the mobile phase is largely determined by the molar concentration of the eluter (counterions). Therefore, the analyte concentration in the eluted fractions is irrelevant to the initial concentration in the sample solution.

Application of highly concentrated solutions tends to modify the solvent composition and interfacial tension, which may lead to a loss of the stationary phase from the column. **Rule 16: If the settling time of the sample solution is lengthy, further dilution of the sample is recommended.** Our experiments have shown that the sample volume can be as large as 200 ml for a separation column of 320 ml capacity, provided that retention of the stationary phase is over 70%.

In the sample solution consisting of two phases, the target analyte should be almost entirely distributed into the stationary phase. If this is not the case, it is most likely that the sample contains a large amount of salt (such as sodium salt), which alters the pH of the sample solution. If this pH shift occurs, retainer should be added to the sample solution to bring the pH into a proper range. Therefore, **Rule 17: Routinely measure the pH of the sample solution before applying it to the column.**

3.5. Separation procedure

The separation is initiated by filling the column entirely with the stationary phase, which is previously either acidified or basified according to the analyte. This is followed by adding the sample solution through the sample port. Then the mobile phase containing the eluter is pumped into the column while the apparatus is rotated at an optimum speed (usually 800 rpm). The effluent is continuously monitored using UV detection followed by on-line pH monitoring (if available) [37] and collected into test tubes using a fraction collector. If an on-line pH monitor is not available, one can manually measure the pH of the collected fractions. Although it is tedious, this manual pH determination has an advantage that it facilitates accurately locating the sharp pH transition point in the collected fractions.

When the hydrophobic counterion, such as DEHPA, is used in the stationary phase, the above operation may result in a steady leakage of a small amount of the counterion, which contaminates the collected fractions. **Rule 18: Leave a small amount of the stationary phase free of hydrophobic counterions at the end of the column.** This is achieved

by partially filling the column with a stationary phase (free of DEHPA but containing the retainer) followed by pumping a measured volume of the stationary phase containing DEHPA. In this way the counterion-free stationary phase retained at the end portion of the column serves as an absorbent for DEHPA.

4. Conclusion

The author hopes that the instructions and advices presented above will be useful and encourage others interested in separations to use this technique which he feels, with

admitted prejudice, has so much to offer them. If questions arise, the author can be reached at itoy2@mail.nih.gov.

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Appendix A

Samples and two-phase solvent systems for high-speed counter-current chromatography

Sample ^a	Solvent systems (volume ratio) ^b	MP ^c	References ^d : (year) page	vol.
Amino acids and peptides				
DNP-amino acids	CHCl ₃ -AcOH-0.1 M HCl (2:2:1)	UP	Science 167 (1970) 281	
Dipeptides	1-BuOH-AcOH-water (4:1:5)	LP	JC 479 (1989) 53	
	MBE-1-BuOH-ACN-water (2:2:1:5)	LP	JCA 702 (1995) 207	
Oligopeptides (anti-HIV)	MBE-ACN-1% TFA (2:2:3)	LP	Mono 1/Ch. 10 (1995) 11	
Bombesin	1-BuOH-DCA-water (100:1:100) (50 °C)	LP	JLC 8 (1985) 2281	
CCK analog	CHCl ₃ -AcOH-water (2:2:1)	LP	JLC 11 (1988) 119	
CCK fragments	1-BuOH-AcOH-water (4:1:5)	LP	JC 484 (1990) 169	
Bovine insulin	1-BuOH-0.5-2% DCA (1:1)	LP	JLC 11 (1988) 55	
Bovine insulin	2-BuOH-1% DCA (1:1)	LP	JC437 (1988) 121	
Antibiotics				
A 201E	CCl ₄ -CHCl ₃ -MeOH-water (2:5:5:5)	UP	JCA 812 (1988) 35	
Actinomycin complex	Hex-Et ₂ O-MeOH-water (1:5:4:5)	UP	JCA 812 (1998) 35	
Bacitracin (peptide Ab)	CHCl ₃ -95% EtOH-water (5:4:3)	LP	JC 538 (1991) 203	
Bacitracin	CHCl ₃ -EtOH-MeOH-water (5:3:3:4)	LP	JC 538 (1991) 203	
Bacitracin	CHCl ₃ -95% EtOH-water (5:4:3)	UP	JCA 812 (1998) 35	
Bacitracin	CHCl ₃ -EtOH-MeOH-water (5:3:3:4)	LP	JCA 812 (1998) 35	
Benzanthrins A and B (quinone Ab)	CCl ₄ -CHCl ₃ -MeOH-water (4:1:4:1)	UP	JCA 812 (1998) 35	
Bu 2313B	<i>n</i> -Hex-CH ₂ Cl ₂ -MeOH-water (5:1:1:1)	LP	JCA 812 (1998) 35	
Candididin (polyene Ab)	CHCl ₃ -MeOH-water (4:4:3)	UP	JCA 812 (1998) 35	
Colistin	1-BuOH-0.04 M TFA (1:1)	LP	JLC 21 (1998) 143	
Coloradocin (macrolide Ab)	CHCl ₃ -MeOH-water (1:1:1)	UP	JCA 812 (1998) 35	
Daunorubicin derivatives	Hex-CHCl ₃ -EtCl-MeOH-water (1:1:1:3.5:1)	UP	JCA 812 (1998) 35	
Doxorubicin/daunorubicin/their metabolites	1-BuOH-0.3 M Na ₂ HPO ₄ (1:1)	LP	JCA 812 (1998) 35	
Dunamycin (macrolide Ab)	Hex-EtOAc-MeOH-water (8:2:10:5)	UP	JCA 812 (1998) 35	

Appendix A (Continued)

Sample ^a	Solvent systems (volume ratio) ^b	MP ^c	References ^d : vol. (year) page
Echinomycin/quinomycin	Hep–EtOAc–Me ₂ CO–water (1:1:3:1)	LP	JCA 812 (1998) 35
Efrotomycin	CCl ₄ –CHCl ₃ –MeOH–water (5:5:6:4)	UP	JCA 812 (1998) 35
Erythromycins A–E	Hex–EtOAc–MeOH–water (1.4:2:2:1)	LP	JLCRT 24 (2001) 1841
Erythromycin	MiBK–Me ₂ CO–0.2 M PCB (pH 6.5) (20:1:21)	LP	JCA 812 (1998) 35
Globoroseamycin (polyene Ab)	CHCl ₃ –MeOH–BB (4:4:3)	LP	JCA 812 (1998) 35
Gramicidin A–C (peptide Ab)	C ₆ H ₆ –CHCl ₃ –MeOH–water (15:15:23:7)	UP and LP	JCA 812 (1998) 35
Ivermectin	Hex–EtOAc–MeOH–water (19:1:10:10)	LP	JCA 723 (1996) 61
Kangudisu/colistin E (peptide Ab)	1-BuOH–2% DCA (5% NaCl) (6:7)	LP	JCA 812 (1998) 35
Mycinamicins	<i>n</i> -Hex–EtOAc–MeOH–8% NH ₄ OH (1:1:1:1)	LP	JCA 812 (1998) 35
Niddamycin (macrolide Ab)	CCl ₄ –MeOH–0.01 M KP (pH 7) (2:3:2)	UP	JCA 812 (1998) 35
Niphimycin	CHCl ₃ –MeOH–water (35:65:40)	LP	JCA 812 (1998) 35
2-Norerythromycin (macrolide Ab)	<i>n</i> -Hep–C ₆ H ₆ –Me ₂ CO–isoPrOH–0.01 M CB (pH 6.3) (5:10:2:3:5)	UP	JLC 11 (1988) 191
2-Norerythromycin	CCl ₄ –MeOH–0.01 M KP (pH 7.3) (1:1:1)	LP	JLC 11 (1988) 191
2-Norerythromycin	CHCl ₃ –MeOH–0.01 M CP (pH 6.0) (1:1:1)	LP	JLC 11 (1988) 191
2-Norerythromycin	Hex–EtOAc–0.01 M KP (pH 7.5) (3:7:5)	UP	JLC 11 (1988) 191
Nystatin	CHCl ₃ –MeOH–BB (2:4:3)	LP	JCA 812 (1998) 35
Oxytetracycline/chlortetracycline	1-BuOH–0.01 M HCl (1:1)	LP	JCA 812 (1998) 35
Pentalenolactone	CHCl ₃ –MeOH–water (1:1:1)	UP	JCA 812 (1998) 35
Pristinamycins (macrolide Ab)	CHCl ₃ –EtOAc–MeOH–water (3:1:3:2)	UP	JCA 812 (1998) 35
Pristinamycins	CHCl ₃ –EtOAc–MeOH–water (2.4:1.6:3:2)	UP	JCA 812 (1998) 35
Siderochelin A	CHCl ₃ –MeOH–water (7:13:8)	UP	JCA 812 (1998) 35
Sporaviridin complex (<i>Streptosporangium viridogriseum</i>)	1-BuOH–Et ₂ O–water (10:4:12)	LP	JLC 13 (1990) 2373
Tetracycline	EtOAc–1-BuOH–0.25 M NH ₄ OAc (1:1:2)	LP	JLC 13 (1990) 2329
Tetracycline	CHCl ₃ –MeOH–PrOH–0.01 M HCl (9:12:1:8)	LP	JLC 7 (1984) 231
Tetracycline/impurities	CH ₃ NO ₂ –CHCl ₃ –pyridine–0.1 M EDTA (pH 7) (20:10:3:33)	LP	JCA 812 (1998) 35
Tiacumicin (macrolide Ab)	CCl ₄ –CHCl ₃ –MeOH–water (7:3:7:3)	UP	JLC 11 (1988) 191
Tiacumicins (macrolide Ab)	CCl ₄ –CHCl ₃ –MeOH–water (7:3:7:3)	UP	JCA 812 (1998) 35
Tirandamycins A and B	<i>n</i> -Hex–EtOAc–MeOH–water (70:30:15:6)	UP	JCA 812 (1998) 35
Trichomycin (polyene Ab)	CHCl ₃ –MeOH–BB (4:4:3)	UP	JCA 812 (1998) 35
Triostin A/echinomycin	TCE–MeOH–water (7:3:1)	UP	JCA 812 (1998) 35
Tyrocidines (peptide Ab)	CHCl ₃ –MeOH–0.1 M HCl (19:19:12)	LP	JCA 812 (1998) 35
WAP-8294-A	1-BuOH–EtOAc–0.005 M TFA (5:15:20)	LP	JCA 932 (2001) 75
Medicinal plants			
Flavonoids			
Isoflavones	EtOAc–1-BuOH–EtOH–water (30:6:10:50)	LP	JCA 992 (2003) 193
	EtOAc–EtOH–water (5:1:5)	LP	JCA 992 (2003) 193
Kaempferol, isorhamnetin quercetin	CHCl ₃ –MeOH–water (4:3:2)	LP	JLCRT 21 (1998) 209

Appendix A (Continued)

Sample ^a	Solvent systems (volume ratio) ^b	MP ^c	References ^d : vol. (year) page
Isoflavones (soybean)	CHCl ₃ –MeOH–water (4:3:2)	LP	JCA 928 (2001) 163
	CHCl ₃ –MeOH–1-BuOH–water (4:3:0.5:2)	LP	JCA 928 (2001) 163
	MBE–THF–BuOH–0.5% TFA (2:2:0.15:4)	LP	JCA 928 (2001) 163
Isoflavones (soybean)	Hex–EtOAc–1-BuOH–MeOH–AcOH–water (1:2:1:1:1:5)	LP	JCA 923 (2001) 271
Isoflavones (soybean)	EtOAc–BuOH–water (2:1:3)	LP	JLCRT 26 (2003) 3487
	Hex–EtOAc–1-BuOH–MeOH–AcOH–water (1:2:1:1:1:5)	LP	JLCRT 26 (2003) 3487
	(2:1:1:3:0.5:5)	LP	JLCRT 26 (2003) 3487
Isoflavones (soybean)	EtOAc–EtOH–water (4:2:7)	LP	JLCRT 26 (2003) 3487
	Hex–EtOAc–MeOH–water (1:1:1:1)	LP	JLCRT 26 (2003) 3487
Flavonoid glycoside	CHCl ₃ –EtOAc–MeOH–water (2:4:1:4)	LP	JC 538 (1991) 219
Salidroside (<i>Rhodiola crenulata</i>)	EtOAc–1-BuOH–water (1:4:5)	LP	JCA 971 (2002) 237
	CHCl ₃ –MeOH–isoPrOH–water (5:6:1:4)	LP	JCA 971 (2002) 237
(±)-Dihydromyricetin (<i>Ampelopsis grssedentata</i>)	Hex–EtOAc–MeOH–water (1:3:2:4)	LP	JCA 973 (2002) 217
Silycristin, silybin, isosilybin (<i>Silybum marianum</i>)	Hex–EtOAc–MeOH–water (1:4:3:4)	LP	JLCRT 25 (2002) 2515
Calycosin (<i>Astragalus membranaceus</i>)	Hex–CHCl ₃ –MeOH–water (1:3:3:2)	LP	JCA 962 (2002) 243
Icariin (<i>Epimedium segittatum</i>)	Hex–1-BuOH–MeOH–water (1:4:2:6)	LP	JCA 962 (2002) 239
Flavonoids (<i>Oroxylum indicum</i>)	Hex–EtOAc–MeOH–water (1:1.2:1:1)	LP	JLCRT 26 (2003) 1623
	(1:1.2:3:1)	LP	JLCRT 26 (2003) 1623
	(0.1:1.6:0.2:1.6)	LP	JLCRT 26 (2003) 1623
Flavonoids from <i>Hippophae rhamnoides</i>	CHCl ₃ –MeOH–water (4:3:2)	LP	JC 445 (1988) 199
Isoflavan and pterocarpan (<i>Astragalus membranaceus</i>)	EtOAc–EtOH–AcOH–water (16:4:1:20)	LP	JCA 1023 (2004) 311
Ginkoflavone glycosides	CHCl ₃ –MeOH–water (4:3:2)	LP	JCA 803 (1998) 268
Daidzin and puerarin	EtOAc–1-BuOH–water (2:1:3)	LP	JCA 855 (1999) 709
3'-Hydroxygenquainin, apigenin and luteolin	CHCl ₃ –MeOH–water (4:3:2)	LP	JC 435 (1988) 159
Catechins: EGCG, ECG and GCG	EtOAc–EtOH–water (25:1:25)	LP	JLCRT 24 (2001) 1723
Catechins: EGCG, ECG and GCG	Hex–EtOAc–water (1:10:10)	LP	JLCRT 24 (2001) 1723
Catechin: EGCG	Hex–EtOAc–water (1:20:30)	LP	JCA 898 (2000) 75
Catechin: EGCG	Hex–EtOAc–MeOH–water (1:1:1:1)	UP	JCA 898 (2000) 75
Catechin: ECG	Hex–EtOAc–water (1:3:4)	LP	JCA 898 (2000) 75
Catechin: ECG	Hex–EtOAc–water (1:9:10)	LP	JCA 27 (2004) 139
Catechins: EGCG and GCG	Hex–EtOAc–water (1:2:3)	LP	JLCRT 21 (1998) 203
(–)-EGC	Hex–EtOAc–water (1:13:20)	LP	JCA 687 (1994) 174
Gallic acid	EtOAc–1-BuOH–water (5:1.8:6)	LP	JCA 886 (2000) 309

Appendix A (Continued)

Sample ^a	Solvent systems (volume ratio) ^b	MP ^c	References ^d : vol. (year) page
Caffeine, EGCG, and epicatechin	Hex–EtOAc–1–BuOH–AcOH–water (0.5:1:2:0.2:6)	LP	AC 72 (2000) 3363
Teaflavines	Hex–EtOAc–MeOH–water (1:3:1:6)	LP	JLCRT 24 (2001) 2363
Apple catechin oligomer	Hex–MeOAc–ACN–water (1:1:1:1)	MidP → UP	JLCRT 26 (2003) 1609
Apple procyanidines	MeOAc–water	UP	JCA 915 (2001) 253
Apple procyanidines	MBE–1–BuOH–ACN–0.1% TFA (4:2:3:8)	LP	JCA 886 (2000) 65
Anthocyanins	MBE–1–BuOH–ACN–water (2:2:1:5) (0.1% TFA)	LP	BEJ 14 (2003) 179
	(1:3:1:5) (0.1% TFA)	LP	BEJ 14 (2003) 179
Alkaloids			
Palmatine, berberine, epiberberine, coptisine, jateorhizine, columbamine (<i>Coptis chinensis</i> Franch)	CHCl ₃ –MeOH–0.2 M HCl (8:3:4)	LP	JCA 829 (1998) 137
Lappaconitine and ranaconitine	CHCl ₃ –MeOH–0.3 M HCl (4:1.5:2)	LP	JCA 943 (2002) 219
Vincamine and vincine	<i>n</i> -Hex–EtOH–water (6:5:5)	LP	JLC 11 (1988) 153
Barringtonine and analogs	CHCl ₃ –0.07 M NaP (pH 5) (1:1)	LP	JLC 15 (1992) 2873
Squalidine, platyphylline neoplatyphylline (<i>Senecio</i>)	CHCl ₃ –0.07 M NaP + 0.04 MCB (pH 6.21–6.45) (1:1)	LP	Mono 1/Ch. 7 (1995) 87
Hydroxygenkwanin, apigenin luteolin [<i>Anisodus tangulicus</i> (Maxin) Pasch]	CHCl ₃ –0.07 M NaP (pH 6.4) (1:1)	UP and LP	JC 435 (1988) 159
Matrine and oxymatrin (<i>Sophora flavescens</i> Ait)	CHCl ₃ –0.07 M NaP (pH 6.4) (1:1)	LP	JLC 13 (1990) 2399
Scopolamine and hyoscyamine (<i>Datura mete</i> L.)	CHCl ₃ –0.07 M NaP (pH 6.5) (1:1)	LP	JLC 13 (1990) 2399
Tetrandrine, fangchinoline, and cyclanoline (<i>Stephania tetrandra</i> S. Moor)	<i>n</i> -Hex–EtOAc–MeOH–water (1:1:1:1)	LP	JLC 11 (1988) 1661
	<i>n</i> -Hex–EtOAc–MeOH–water (3:7:5:5)	LP	JLC 11 (1988) 1661
Anthraquinones			
Hydroxyanthraquinones (<i>Rheum palmatum</i> L.)	<i>n</i> -Hex–EtOAc–MeOH–water (9:1:5:5)	UP → LP	JC 442 (1988) 455
Rhein, chrysophanol, emodin, aloemodin, physcion (<i>Rheum officinale</i> Baill)	Et ₂ O–4.0% NaHCO ₃ → 0.7% Na ₂ CO ₃ → 0.2% NaOH (1:1)	LP	JCA 858 (1999) 103
Resveratrol A and aglycoside B (<i>Polygonum cuspidatum</i>)	CHCl ₃ –MeOH–water (4:3:2)	LP	JCA 919 (2001) 443
Lignans			
Schisanherol and its acetate (<i>Schisandra rubriflora</i> Rhed and Wils)	<i>n</i> -Hex–EtOAc–MeOH–water (10:5:5:1)	UP ⇌ LP	JNP 52 (1989) 706

Appendix A (Continued)

Sample ^a	Solvent systems (volume ratio) ^b	MP ^c	References ^d : vol. (year) page
Anti-HIV lignans (<i>Larria tridentata</i>)	Hex–EtOAc–MeOH–0.5% NaCl (7:3:5:5)	UP	JCA 719 (1996) 353
	(7:3:5:5)	UP	JCA 719 (1996) 353
Rhein (<i>Rheum officinale</i> Baill)	Hex–CHCl ₃ –MeOH–1.2% NaCl (1:4:4:2)	LP	JCA 719 (1996) 353
	<i>n</i> -Hex–EtOAc–MeOH–water (3:7:5:5)	LP	JCA 1017 (2003) 125
Steroids			
25-Hydroxycholecalciferol	Hex–EtOAc–MeOH–water (5:1:5:1)	LP	JLC 18 (1995) 181
Przewaquinone A (<i>Salvia miltiorrhiza</i>)	Hex–CCl ₄ –MeOH–water (1:3:3:2)	LP	JLCRT 26 (2003) 1267
Cucurbitacin B and E (<i>Cucumis mel</i> L.)	Hex–EtOAc–MeOH–water (12:24:16:9)	UP	Mono 1/Ch. 9 (1995) 107
Quadri-terpenic acids (<i>Ligustrum lucidum</i>)	Hex–EtOAc–MeOH–water (3:6:2:1)	LP	JLC 18 (1995) 1997
Triterpenic acid (<i>Boswellia carterii</i>)	<i>n</i> -Hex–EtOH–water (6:5:2)	LP	JLC 13 (1990) 2389
Steroid alcohols (<i>Tieghemella heckelli</i>)	MBE–1-BuOH–ACN–0.5% TFA (1:3:3:4)	LP	JLCRT 25 (2002) 2871
Saponins			
Dammarane-saponins (<i>Panax notoginseng</i>)	CHCl ₃ –MeOH–2-BuOH–water (5:6:1:4)	LP	JLCRT 26 (2003) 1579
	EtOAc–1-BuOH–water (1:1:2)	LP	JLCRT 26 (2003) 1579
Arganines and tieghemelin (<i>Tieghemella heckelli</i>)	MBE–1-BuOH–ACN–0.5% TFA (1:3:1:5)	LP	JLCRT 26 (2002) 3197
Miscellaneous			
Taxol and cephalomannine	Hex–EtOAc–MeOH–water (6:4:5:5)	LP	JLCRT 21 (1998) 157
10-Deacetylbaicatin III	Hex–EtOAc–EtOH–water (2:5:2:5)	LP	JCA 813 (1998) 397
Artemisinin, artemisitene and arteannuin B	Hex–EtOAc–EtOH–water (8:2:5:4)	LP	JLCRT 23 (2000) 909
	Hex–EtOAc–EtOH–water (6:4:5:4)	LP	JLCRT 23 (2000) 909
Notopterol and isoimperatorin	LtPet–EtOAc–MeOH–water (5:5:5:4)	LP	JCA 883 (2000) 67
	LtPet–EtOAc–MeOH–water (5:5:4.8:5)	LP	JCA 883 (2000) 67
Stilbene glycoside (<i>Poligonum multiflorum</i>)	EtOAc–EtOH–water (50:1:50)	LP	JLCRT 21 (1998) 2897
Acteoside and 2'-acetylacteoside (<i>Cistanches salsa</i>)	EtOAc–1-BuOH–EtOH–water (20:3:3:25)	LP	JCA 912 (2001) 181
Acteoside and isoacteoside	EtOAc–1-BuOH–EtOH–water (35:6:6:50)	LP	JLCRT 24 (2001) 2127
	EtOAc–1-BuOH–EtOH–water (30:10:6:50)	LP	JLCRT 24 (2001) 2127
Mevinolinic acid (<i>Monascus purpureus</i>)	<i>n</i> -Hex–EtOAc–MeOH–water (1:1:1:1)	LP	JLCRT 26 (2003) 3083
Taxol and cephalomannine	<i>n</i> -Hex–EtOAc–MeOH–water (6:3:5:5)	LP	JLCRT 21 (1998) 157
Strychnine (<i>Strychnos nux-vomica</i> L.)	CHCl ₃ –0.07 M NaP (pH 5.08) (1:1)	LP	JLCRT 21 (1998) 157
Triptolidides (<i>Tripterygium wilfordii</i>)	Hex–CH ₂ Cl ₂ –MeOH–water (3:22:17:8)	LP	JLCRT, in press

Appendix A (Continued)

Sample ^a	Solvent systems (volume ratio) ^b	MP ^c	References ^d : vol. (year) page
Dyes and pigments			
Gardenia yellow	EtOAc–1-BuOH–water (2:3:5)	UP	Mono 1/Ch. 8 (1995) 92
Methyl violet 2B	CHCl ₃ –AcOH–0.1 M HCl (2:2:1)	UP	AC 57 (1985) 376
Tetrabromofluorescein and Phloxine B	EtOAc–1-BuOH–0.01 M NH ₄ OAc (1:1:2)	UP and LP	JC 538 (1991) 157
Isomeric sulfophthalic acids	1-BuOH–water–HCl (6:6:0.045) (pH 1.2)	LP	JCA 966 (2002) 111
Apocartinoids (<i>Cochlospermum tinctorium</i>)	CCl ₄ –MeOH–water (5:4:1)	UP	JLC 11 (1988) 227
Azo dyes	1-BuOH–pyridine–water (10:7:3)	LP	JLC 11 (1988) 251
Lac dye	Hex–EtOAc–MeOH–water (2:2:1:5)	LP	JCA 813 (1998) 71
Lipids			
Steroid intermediates	<i>n</i> -Hex–EtOH–water (6:5:4)	UP ⇌ LP	JLC 11 (1988) 37
Steroids	<i>n</i> -Hex–EtOAc–MeOH–water (6:5:5:5)	UP ⇌ LP	JLC 11 (1988) 37
Progesterone	Hex–MeOH–water (6:5:3)	LP	AC 72 (2000) 3363
Unsaturated fatty acids	<i>n</i> -Hex–CAN	UP → LP	JLC 11 (1988) 283
Free fatty acids	Hep–ACN–AcOH–MeOH (4:5:1:1)	LP	JCA 1021 (2003) 117
Fish oil ethyl esters	Hex–CHCl ₃ –ACN (5:1:4)	LP	JLCRT 19 (1996) 1451
Tanshinones	Hex–EtOAc–water (4:1.8:2) → (4:2.3:2) → (4:3:2)	LP	JCA 904 (2000) 107
Tanshinones	LtPet–EtOAc–MeOH–water (2:3:2.5:1.7)	LP	JCA 945 (2002) 281
Neutral phospholipids	Hex–EtOAc–EtOH–water (5:5:5:4)	UP and LP	JLCRT 25 (2002) 1255
Glycoglycerolipids	Hex–EtOAc–EtOH–water (3:5:3:4)	UP and LP	JLCRT 25 (2002) 1255
Glycolipids	Hex–EtOH–water (5:4:1)	LP	JLCRT 21 (1998) 103
Agrochemicals			
Indole auxins (ASA, IAcA, IBA, ICA, IPA)	CHCl ₃ –AcOH–water (2:2:1)	UP	JC 247 (1982) 315
Indole auxins (IA, IAA, ICA, IBA)	<i>n</i> -Hex–EtOAc–MeOH–water (3:7:5:5)	LP	JLC 11 (1988) 75
Zeatin, zeatin riboside, 6-isophenyl adenosine and 6-isophenyl adenine	EtOAc–MeOH–0.5 M KP (pH 7) (3:1:3)	LP	JC 247 (1982) 315
<i>S</i> -Triazine herbicide	<i>n</i> -Hex–EtOAc–MeOH–water (8:2:5:5)	UP	JLC 11 (1988) 75
Azadirachtin	<i>n</i> -Hex–EtOAc–MeOH–water (3:5:3:5)	LP	MFLG 62 (1997) 213
20-Hydroxyecdysone and ajugasterone C (<i>Vitex madiensis</i>)	CHCl ₃ –MeOH–water (13:7:4)	UP	JLC 11 (1988) 173
Abscisic acid derivatives	MBE–1-BuOH–ACN–water (1:3:1:5)	LP	Phyt 65 (2004) 9955
Vitamins			
Thiamine nitrate, riboflavine pyridoxine HCl, nicotine amide	1-BuOH–EtOH–0.15 M KH ₂ PO ₄ (8:3:8)	LP	JLCRT 23 (2000) 1403
Calciferol, V–A acetate, (±)- α -tocopherol acetate, V–K	Isooctane–MeOH	LP	JLCRT 23 (2000) 1403
Lycopene (tomato paste)	Hex–CH ₂ Cl ₂ –ACN (20:7:13)	LP	JCA 929 (2001) 169

Appendix A (Continued)

Sample ^a	Solvent systems (volume ratio) ^b	MP ^c	References ^d : vol. (year) page
Lutein (marigold flower)	<i>n</i> -Hex–CHCl ₃ –ACN (10:3:7)	UP	JLCRT 26 (2003) 1659
Various organic acids			
Hippuric acid, mandelic acid and benzoic acid	MBE–0.1% TFA (1:1)	UP and LP	JLCRT 23 (2000) 1575
2-(2'-Hydroxyethoxy) terephthalic acid	CHCl ₃ –MeOH–water (37:37:26)	UP	JLC 11 (1988) 245
Naphthalene, benzophenone, <i>o</i> -nitrophenol and acetophenone	<i>n</i> -Hex–MeOH–water (3:3:2)	LP	JLC 11 (1988) 91
Acetophenone, benzoic acid, <i>p</i> -nitrophenone and phenol	CHCl ₃ –MeOH–HCl (pH 2) (3:1:3)	LP	JLC 11 (1988) 91
Bromoacetyl-T ₃	Hex–EtOAc–MeOH–15 mM NH ₄ OAc (pH 4) (1:1:1:1) and (4:5:4:5)	LP	JC 538 (1991) 165
Sugars and PNP derivatives			
PNP glucose	EtOAc–1-BuOH–water (1:4:5)	LP	JLCRT 22 (1999) 579
Sucrose and fucose	1-BuOH–AcOH–water (4:1:5)	LP	JLCRT 22 (1999) 579
Glucuronic acid	1-BuOH–EtOH–water (4:1:4)	LP	JLCRT 22 (1999) 579
Inorganic elements			
Rare earth elements (La, Ce, Pr, Nd, Sm, Eu)	0.1 M DEHPA in Hep–0.03–0.15 M HCl (1:1)	LP	JLC 11 (1988) 267
Rare earth elements	0.02 M DEHPA in kerosene–0.1 M DCA in 20% EG–water (pH 1.35–2.5) (1:1)	LP	JLC 11 (1988) 2517
LaCl ₃ , PrCl ₃ , NdCl ₃	0.02 M DEHPA in Hep–0.02 M HCl (1:1)	LP	JLC 13 (1990) 2329
La, Pr and Nd	0.02 M DEHPA in Hep–0.02 M HCl (1:1)	LP	JC 538 (1991) 133
12 Rare earth elements	0.003 M DEHPA in Hep–0–0.3 M HCl (1:1)	LP	JC 538 (1991) 133
Heavy metals (Ca, Cd, Co, Fe(III), Mg, Mn, Ni, P, Pb, Zn)	0.6 M DEHPA in Hep–0.1 M TA (1:1)	LP	JCA 21 (1998) 251

^a Ab: antibiotics; ABC: abscisic acid; DNP: dinitrophenyl; CCK: cholecystokinin; ECG: epicatechin-3-gallate; EGC: epigallocatechin; EGCG: epigallocatechin-3-*O*-gallate; GCG: gallic acid-3-*O*-gallate; IA: indole-3-acetamide; IAA: indole-3-acetic acid; IAcA: indole-3-acrylic acid; IBA: indole-3-butyric acid; ICA: indole-3-carboxylic acid; T₃: 3,3',5-triiodo-L-thyronine; PNP: *p*-nitrophenyl.

^b AcOH: acetic acid; BB: borate buffer; BuOH: butanol; CB: citrate buffer; CP: citrate + phosphate buffer; DCA: dichloroacetic acid; DEHPA: di(2-ethylhexyl)phosphoric acid; EDTA: ethylenediaminetetraacetic acid; EG: ethylene glycol; EtCl: ethylenechloride, EtOAc: ethyl acetate; EtOH: ethanol; Et₂O: diethyl ether; Hep: heptane; Hex: hexane; KP: potassium phosphate; LtPet: light petroleum; MBE: methyl *tert*-butyl ether; Me₂CO: acetone; MeOAc: methyl acetate; MeOH: methanol; NaP: sodium phosphate; NH₄OAc: ammonium acetate; PCB: phosphate + citric acid buffer; PEG: polyethylene glycol; PrOH: propanol; TA: tartaric acid; TFA: trifluoroacetic acid; THF: tetrahydrofuran.

^c LP: lower phase; MP: mobile phase; MidP: middle phase; UP: upper phase; UP → LP: UP followed by LP (reversed elution mode); UP ⇌ LP: dual CCC.

^d AC: Analytical Chemistry; BEJ: Biochemical Engineering Journal; JC: Journal of Chromatography; JCA: Journal of Chromatography A; JLC: Journal of Liquid Chromatography; JLCRT: Journal of Liquid Chromatography and Related Technologies; JNP: Journal of Natural Products; MFLG: Medical Fac. Landbouww. Univ. Gent; Mono 1: ACS Monograph on Modern Counter-current Chromatography (Editors: W.D. Conway and R.J. Petroski) 1995; Phyt: Phytochemistry.

Appendix B

Samples and two-phase solvent systems for pH-zone-refining counter-current chromatography

Sample ^a (mass)	Solvent systems ^b (volume ratio)	Key reagent ^c		References ^d vol. (year) page
		Retainer in SP	Eluter in MP	
Amino acids and peptides				
DNP–amino acids (0.3 g)	MBE–CAN–water (4:1:5)	TFA (0.04%/UP)	NH ₃ (0.1%/LP)	JACS 116 (1994) 704
DNP–amino acids (0.7 g)	MBE–water	NH ₃ (22 mM/LP)	TFA (5–20 mM/UP)	JCA 672 (1994) 101
DNP–amino acids (1 g)	MBE–CAN–water (4:1:5)	TFA (200 μl/SS)	NH ₃ (0.1%/LP)	Mono 1/Ch. 14 (1995) 154
Amino acid–OBzl (0.7 g)	MBE–water	TEA (2.5–40 mM/UP)	HCl (5–40 mM/LP)	JCA 678 (1994) 233
Proline–OBzl (1 g)	MBE–water	TEA (10 mM/UP)	HCl (10 mM/LP)	Mono 1/Ch. 14 (1995) 154
<i>N</i> -CBZ–dipeptides (0.8 g)	MBE–CAN–water (2:2:3)	TFA (16 mM/UP)	NH ₃ (5.5 mM/LP)	JCA 702 (1995) 197
<i>N</i> -CBZ–tripeptides (0.5 g)	MBE–CAN–1- BuOH–water (2:2:1:5)	TFA (16 mM/UP)	NH ₃ (2.7 mM/LP)	JCA 702 (1995) 197
Dipeptide–βNA (0.3 g)	MBE–ACN–water (2:2:3)	TEA (5 mM/UP)	HCl (5 mM/LP)	JCA 702 (1995) 197
Dipeptide (2 g)	MBE–ACN–water (4:1:5)	TFA (20 mM/UP) + DEHPA (30–40%/UP)	HCl (20 mM/LP)	JCA 771 (1997) 81
Bacitracin (5 g)	MBE–CAN–water (4:1:5)	TFA (40 mM/UP)	HCl (20 mM/LP)	JCA 771 (1997) 81
Bovine insulin (0.2 g)	MBE–water	TFA (0.04%/UP)	NH ₃ (0.1%/LP)	Mono 1/Ch. 14 (1995) 154
Alkaloids				
Crinine, powelline and crinamidine (3 g)	MBE–water	TEA (5 mM/UP)	HCl (5 mM/LP)	JCA 685 (1994) 259
	MBE–water	HCl (10 mM/LP)	TEA (10 mM/UP)	JCA 685 (1994) 259
Matrin and sophocarpine (2 g)	MBE–water	TEA (10 mM/UP)	HCl (5–10 mM/LP)	JCA 822 (1998) 316
Lappaconitine (10.5 g)	MBE–THF–water (2:2:3)	TEA (10 mM/UP)	HCl (10 mM/LP)	JCA 923 (2001) 281
Vincine and vincamine (0.3 g)	MBE–water	TEA (5 mM/UP)	HCl (5 mM/LP)	JCA 753 (1996) 1
Catecholamines (3 g)	MBE–water	NH ₄ OAc (200 mM/UP) + DEHPA (20%/UP)	HCl (50 mM/LP)	JCA 724 (1996) 348
Dyes				
D&C Orange No. 5 (5 g)	DEE–ACN–10 mM NH ₄ OAc (4:1:5)	TFA (0.2 ml/SS)	NH ₃ (LP/pH 9)	JACS 116 (1994) 704

Appendix B (Continued)

Sample ^a (mass)	Solvent systems ^b (volume ratio)	Key reagent ^c		References ^d vol. (year) page
		Retainer in SP	Eluter in MP	
D&C Red No. 3 (3 g)	DEE–ACN–10 mM NH ₄ OAc (4:1:5)	TFA (0.4 ml/500 ml UP)	NH ₃ (LP/pH 7.5)	JCA 658 (1994) 505
D&C Red No. 28 (3 g)	DEE–ACN–10 mM NH ₄ OAc (4:1:5)	TFA (0.6 ml/500 ml UP)	NH ₃ (LP/pH 8.1)	JCA 678 (1994) 77
D&C Red No. 28 (6 g)	DEE–ACN–10 mM NH ₄ OAc (4:1:5)	TFA (1.2 ml/SS)	NH ₃ (LP/pH 9.2)	JCA 678 (1994) 77
D&C Red No. 22 (5 g)	DEE–ACN–10 mM NH ₄ OAc (4:1:5)	TFA (0.8 ml/500 ml UP)	(0.01%/LP)	Mono 2/Ch. 12 (1996) 337
TCF (1 g)	DEE–ACN–10 mM NH ₄ OAc (4:1:5)	TFA (0.25 ml/SS)	NH ₃ (LP/pH 9.3)	Mono 1/Ch. 16 (1995) 203
TCF (5 g)	MBE–ACN–water (4:1:5)	TFA (0.2 ml/500 ml UP)	NH ₃ (LP/pH 10.7)	Mono 1/Ch. 16 (1995) 203
Brominated TCF (5 g)	DEE–ACN– water (4:1:5)	TFA (24.7 mM/UP)	NaOH (65 mM/LP)	JCA 732 (1996) 283
Rose bengal (1.5 g)	DEE–ACN– water (4:1:5)	TFA (5 mM/UP)	NH ₃ (19.8 mM/LP)	Mono 2/Ch. 12 (1996) 337
Yellow No. 203 (5 g)	isoAmOH–MBE– ACN–water (3:5:1:7)	DA (20%/UP) + H ₂ SO ₄ (40 mmol/SS)	NH ₃ (163 mM/LP)	EncSS 6 (2000) 2588
D&C Yellow No. 10 (1.6 g)	isoAmOH–MBE– ACN–water (3:1:1:5)	DA (275 mM/UP) + H ₂ SO ₄ (37.7 mM/UP)	NH ₃ (80 mM/LP)	JCA 923 (2001) 87
Red No. 106 (0.3 g)	1-BuOH–water	H ₂ SO ₄ (40 mM/UP)	NH ₃ (30 mM/LP)	JCA 946 (2002) 157
FD&C Yellow No. 6 (2 g)	MBE–ACN–water (4:1:5)	H ₂ SO ₄ (0.2%/UP) + TDA (5%/UP)	NH ₃ (0.4%/LP)	JCA 753 (1996) 1
Isomers				
MMCCA (0.4 g)	Hex–EtOAc–MeOH– water (1:1:1:1)	TFA (1.3 mM/UP) + octanoic acid (3.4 mM/UP)	NH ₃ (3.4 mM/LP)	JCA 685 (1994) 253
NCMBA (10 g)	MBE–ACN–water (4:1:5)	TFA (12 mM/UP)	NH ₃ (100 mM/LP)	JLCRT 21 (1998) 195
NACBA (15 g)	MBE–ACN–water (4:1:5)	TFA (0.32%/UP)	NH ₃ (0.8%/LP)	JLC 17 (1994) 3507
(±)-DNB–leucine (2 g)	MBE–water	TFA (40 mM/UP) + DPA (40 mM/UP)	NH ₃ (20 mM/LP)	JCA 704 (1995) 75
(±)-DNB–valine (2 g)	MBE–water	TFA (40 mM/UP) + DPA (40 mM/UP)	NH ₃ (20 mM/LP)	JCA 753 (1996) 1
3- and 4-Sulfophthalic acids (10 g)	1-BuOH–water	HCl (488 mM/UP)	NH ₃ (105 mM/LP)	JCA 966 (2002) 111
Miscellaneous				
Coumarin (0.3 g)	MBE–water	TFA (10 mM/UP)	NH ₃ (20 mM/LP)	JCA 759 (1997) 47
NDGA (20 g)	MBE–water	TFA (25 mM/UP)	NaOH (100 mM/LP)	JLCRT 21 (1998) 171

Appendix B (Continued)

Sample ^a (mass)	Solvent systems ^b (volume ratio)	Key reagent ^c		References ^d vol. (year) page
		Retainer in SP	Eluter in MP	
Curcumin (20 g)	MBE–ACN–water (4:1:5)	TFA (20 mM/UP)	NH ₃ (30 mM/LP)	JLCRT 23 (2000) 2209
Indole auxins (0.8 g)	MBE–water	TFA (0.04%/UP)	NH ₃ (0.05%/LP)	JCA 753 (1996) 1
Fucans (0.8 g)	10% LA in MiBK–25 mM NaOH	–	–	JCB 706 (1998) 43

^a DNP: dinitrophenyl; CBZ: carbobenzoxy; βNA: naphthylamine; TCF: tetrachlorofluorescein; MMCCA: 1-methyl-4-methoxymethyl cyclohexane carboxylic acid; NCMBA: 2- and 6-nitro-4-chloro-3-methoxybenzoic acid; NACBA: 2- and 6-nitro-3-acetamido-4-chlorobenzoic acid; DNB: dinitrobenzoyl; NDGA: nordihydroguaiaretic acid.

^b MBE: methyl *tert*-butyl ether; ACN: acetonitrile; BuOH: butanol; THF: tetrahydrofuran; DEE: diethyl ether; NH₄OAc: ammonium acetate; AmOH: amyl alcohol; Hex: hexane; EtOAc: ethyl acetate; MeOH: methanol; MiBK: methyl isobutyl ketone; LA: liquid amberlite.

^c SP: stationary phase; MP: mobile phase; UP: upper phase; LP: lower phase; SS: sample solution; TFA: tetrafluoroacetic acid; TEA: triethylamine; DEHPA: di(2-ethylhexyl)phosphoric acid; DA: dodecylamine; EtOAc: ethyl acetate; TDA: tridodecylamine; DPA: *N*-dodecanoyl-L-proline-3,5-dimethylamide (chiral selector).

^d JACS: Journal of American Chemical Society; EncSS: Encyclopedia of Separation Science; JCA: Journal of Chromatography; JLC: Journal of Liquid Chromatography; JLCRT: Journal of Liquid Chromatography and Related Technologies; Mono 1: ACS Monograph on Modern Counter-current Chromatography, 1995 (Editors: W.D. Conway and R.J. Petroski) (Ref. [10]); Mono 2: High-Speed Countercurrent Chromatography, 1996 (Editors: Y. Ito and W.D. Conway) Wiley (Ref. [11]).

References

- [1] Y. Ito, R.L. Bowman, *Science* 167 (1970) 281.
- [2] Y. Ito, R.L. Bowman, *J. Chromatogr. Sci.* 8 (1970) 315.
- [3] T. Tanimura, J.J. Pisano, Y. Ito, R.L. Bowman, *Science* 169 (1970) 54.
- [4] Y. Ito, *J. Chromatogr.* 214 (1981) 122.
- [5] Y. Ito, J.L. Sandlin, W.G. Bowers, *J. Chromatogr.* 244 (1982) 247.
- [6] Y. Ito, W.D. Conway, *Anal. Chem.* 56 (4) (1984) 534A.
- [7] Y. Ito, *Adv. Chromatogr.* 24 (1984) 181.
- [8] Y. Ito, in: N.B. Mandava, Y. Ito (Eds.), *Countercurrent Chromatography: Theory and Practice*, Marcel Dekker, New York, 1988, pp. 79–442.
- [9] W.D. Conway, *Countercurrent Chromatography: Apparatus, Theory and Applications*, VCH, New York, 1990.
- [10] W.D. Conway, R.J. Petroski (Eds.), *Modern Countercurrent Chromatography* (ACS Symposium Series No. 593), American Chemical Society, Washington, DC, 1995.
- [11] Y. Ito, W.D. Conway (Eds.), *High-speed Countercurrent Chromatography*, Wiley-Interscience, New York, 1996.
- [12] J.-M. Menet, D. Thiébaud (Eds.), *Countercurrent Chromatography*, Marcel Dekker, New York, 1999.
- [13] A. Berthod (Ed.), *Countercurrent Chromatography*, Elsevier, Amsterdam, 2003.
- [14] Y. Ito, in: E. Heftmann (Ed.), *Chromatography V, Part A, Chapter 2*, Journal of Chromatography Library, Elsevier, Amsterdam, 1992, pp. A69–A107.
- [15] Y. Ito, in: A. Townshend, G. Fullerlove (Eds.), *Encyclopedia of Analytical Science*, vol. 2, Academic Press, London, 1995, pp. 910–916.
- [16] Y. Ito, in: J. Cazes (Ed.), *Encyclopedia of Chromatography*, Marcel Dekker, New York, 2001, pp. 438–440.
- [17] Y. Ito, *CRC Crit. Rev. Anal. Chem.* 17 (1986) 65.
- [18] Y. Ito, *J. Liq. Chromatogr.* 15 (1992) 2639.
- [19] H. Tsugita, *Zikken Kagaku Koza 2 (Kisogijutsu II)*, Maruzen, Tokyo, 1956, pp. 303–361, Chapter 9 (in Japanese).
- [20] L.C. Craig, *Comprehensive Biochemistry*, vol. 4, Elsevier, Amsterdam, 1962.
- [21] D.G. Martin, in: W.D. Conway, R.J. Petroski (Eds.), *Modern Countercurrent Chromatography* (ACS Symposium Series, No. 593), American Chemical Society, Washington, DC, 1995 (Chapter 10).
- [22] J.H. Renault, J.M. Nuzillard, O. Intes, A. Maciuk, in: A. Berthod (Ed.), *Countercurrent Chromatography*, Elsevier, Amsterdam, 2003 (Chapter 3).
- [23] F. Oka, H. Oka, Y. Ito, *J. Chromatogr.* 538 (1991) 99.
- [24] Y. Ito, W.D. Conway, *J. Chromatogr.* 301 (1984) 405.
- [25] Q.-Z. Du, C.-J. Wu, J.-G. Qian, P.-D. Wu, Y. Ito, *J. Chromatogr. A* 875 (1999) 231.
- [26] D.E. Schaufelberger, T.G. McCloud, J.A. Beutlér, *J. Chromatogr.* 538 (1991) 87.
- [27] H. Oka, Y. Ito, in: J. Cazes (Ed.), *Encyclopedia of Chromatography*, Marcel Dekker, New York, 2001, pp. 208–211.
- [28] F.-Q. Yang, Y. Ito, *J. Liq. Chromatogr. Rel. Technol.* 27 (2004) 2979.
- [29] A. Weisz, A.L. Scher, K. Shinomiya, H.M. Fales, Y. Ito, *J. Am. Chem. Soc.* 116 (1994) 704.
- [30] Y. Ito, Y. Ma, *J. Chromatogr. A* 753 (1996) 1.
- [31] Y. Ito, K. Shinomiya, H.M. Fales, A. Weisz, A.L. Scher, in: W.D. Conway, R.J. Petroski (Eds.), *ACS Monograph on Modern Countercurrent Chromatography*, 1995, p. 154, Chapter 14.
- [32] Y. Ito, in: J. Cazes (Ed.), *Encyclopedia of Chromatography*, Marcel Dekker, New York, 2001, pp. 606–611.
- [33] A. Weisz, Y. Ito, in: I.D. Wilson, E.R. Adlard, M. Cooke, C.F. Poole (Eds.), *Encyclopedia of Separation Science*, vol. 6 (III), Academic Press, London, 2000, pp. 2588–2602.
- [34] Y. Ito, Y. Shibusawa, H.M. Fales, H.J. Cahnman, *J. Chromatogr.* 625 (1992) 177.
- [35] Y. Ma, Y. Ito, A. Foucault, *J. Chromatogr. A* 704 (1995) 75.
- [36] Y. Ma, Y. Ito, in: J. Cazes (Ed.), *Encyclopedia of Chromatography*, Marcel Dekker, New York, 2001, pp. 160–163.
- [37] A. Weisz, A.L. Scher, Y. Ito, *J. Chromatogr. A* 732 (1996) 283.

Glossary

Archimedean screw effect (head and tail): when the coiled column filled with two solvent phases is subjected to a planetary motion (rotating centrifugal force field), both phases tend to advance toward one end (head) of an open coil determined by the Archimedean screw. However, in a closed coil type-J synchronous planetary motion gives an advantage for the lighter phase to occupy the head end while the heavier phase is pushed back toward the other end called the tail. This phenomenon is efficiently used for retaining the stationary phase by either introducing the heavier phase from the head or the lighter phase from the tail into the rotating coiled column filled with the other phase as the stationary phase.

Carryover of the stationary phase: when the mobile phase is eluted through the column previously filled with the stationary phase, it gradually displaces the stationary phase until it elutes out from the column (solvent front) and the hydrodynamic equilibrium is established through the column. If the stationary phase keeps flowing out from the column after the emergence of the solvent front, it is called carryover of the stationary phase or stripping. Usually this tapers off shortly after the solvent front. Continued carryover of the stationary phase not only significantly reduces the resolution, but also disturbs the tracing of the elution curves. This complication may be caused by various reasons including a high flow rate of the mobile phase; long *settling time* of the solvent system (over 20 s) especially when the upper phase is eluted from the tail toward the head; emulsification of the sample compartment when the sample contains detergent-like compounds such as saponin; precipitate formation in the column during pH-zone-refining CCC, etc.

Chiral CCC: enantiomers can be resolved if a suitable chiral selector is dissolved in the stationary phase. For example, *N*-dodecanoyl-L-proline-3,5-dimethylanilide is effectively used for resolving (\pm)-dinitrobenzoyl-amino acids.

Column capacity (V_C): the total volume of the separation column. In a typical commercial HSCCC, the column capacity ranges from 100 ml (analytical) to 1 l (preparative).

Counter-current chromatography (CCC): continuous liquid–liquid partition technique without a solid support. The method employs variety of partition systems using either gravitational or centrifugal force to retain the stationary phase in the column. The method allows continuous elution of the mobile phase and on-line monitoring of the effluent as in liquid chromatography. High-speed CCC (HSCCC) is described below.

Counter-current distribution (CCD) method: a discontinuous extraction method using a set of multiple partition units. The method was rather widely used for purification of natural products during the 1950s. The Craig apparatus is the most advanced form using hundreds of complex glass partition units connected in series. Each partition process consists of three steps, i.e., mixing, settling and transfer of the upper mobile phase to the next unit containing the lower stationary phase.

Degassing of two-phase solvent system: HSCCC produces a pressure gradient from the head toward the tail by an Archimedean screw force and a drag of the mobile phase flow. Although the pressure drop in HSCCC is usually no more than a few hundred p.s.i., it often causes bubble formation in the outlet where the pressure drops to an atmospheric level. Therefore, it is desirable to eliminate air from the two-phase solvent system before application to the separation column. This can be done in a separatory funnel as follows: after each shaking the contents, invert the funnel and open the stopcock to void the air. This procedure should be repeated at least five times. When the lighter upper phase is used as the mobile phase (tail to head elution mode which tends to produce negative pressure in the column), the degassing should be more completely performed by connecting the outlet of the inverted funnel to a vacuum line once or twice. The cock should be opened only momentarily to avoid a loss of the organic solvent.

Eluter: see retainer and eluter.

Elution mode: this term indicates how to introduce the mobile phase (upper or lower) into the rotating coil (from the head or the tail). Of four combinations, only two elution modes should be used in HSCCC, i.e., either the lower phase from the head toward the tail or the upper phase from the tail toward the head.

Head and tail of the rotating coil: see Archimedean screw effect.

High-speed CCC (HSCCC) (type-J): the most advanced form of the CCC system using a multilayer coil separation column, which undergoes a type-J synchronous planetary motion (see Fig. 1). The method covers analytical (microgram) to preparative (10 g) scale separations usually in a few hours at a high partition efficiency up to a few thousand theoretical plates.

Hydrodynamic equilibrium: when the column filled with the stationary phase is eluted with the mobile phase, the planetary motion produces an Archimedean screw effect, which moves the upper phase toward the head of the coil. If the mobile phase is the lower phase eluted from the head toward the tail, the movement of the stationary upper phase toward the head is offset by the flow of the mobile lower phase toward the tail, resulting in a steady hydrodynamic balance between the two phases where a given amount of the stationary phase is permanently retained in the coil. In this case, a pressure gradient is formed from the head toward the tail (outlet) where the pressure drops down to near the atmospheric level. This hydrodynamic state is quite stable as far as the flow rate of the mobile phase is within a reasonable range. On the other hand, if the upper phase is pumped into the tail of the coil filled with the lower phase, both the Archimedean Screw effect and the flow of the mobile phase pushes two phases toward the outlet (the upper phase moves faster than the lower phase through the coil) producing a decreasing pressure gradient from the head (outlet) toward the tail (inlet). This hydrodynamic condition often creates a negative pressure at the inlet of the column which tends to suck an extra amount of the mobile phase from the reservoir through a pair of check valves of the metering pump, increasing the flow rate of the mobile phase. Negative column pressure also tends to cause a formation of air bubbles in the column causing the problem in recording the elution curve. However, these problems can be eliminated by applying a piece of narrow tubing at the outlet of the monitor to raise the inlet column pressure to a positive range. Also, when the *settling time* of the solvent is over 20 s, this hydrodynamic condition tends to cause a gradual loss of the stationary phase from the column (carryover). Therefore, if there is a choice of the mobile phase, it is safer to use the lower phase as the mobile phase pumping it from the head of the coiled column. It must be mentioned, however, that if the *settling time* is short (less than 10 s) and the partition coefficient is suitable, the upper mobile phase will produce an excellent separation, provided that the inlet column pressure is always kept above the atmospheric level as mentioned earlier.

log P : partition coefficient of drugs measured by a binary two-phase solvent system composed of *n*-octanol–water and expressed in a logarithmic scale. It indicates hydrophobicity of the drug where a higher log P value is considered to be more effective.

Mobile phase: one of the two solvent phases, either upper or lower, which elutes through the column.

Multilayer coil: a separation column used for HSCCC. It is prepared from a long piece of polytetrafluoroethylene (PTFE) tubing by coaxially winding it around a spool-shaped holder making multiple coiled layers. Usually three different sizes of tubing are used: ca. 1 mm i.d. for analytical, 1.6 mm i.d. for semipreparative, and 2.6 mm i.d. for preparative separations. It provides efficient mixing of the two phases and a high retention of the stationary phase under a high flow rate of the mobile phase.

Non-linear isotherm: isotherm is a line drawn on a graph paper by plotting the solute concentration in the upper phase against that in the lower phase in an equilibrated two-phase solvent system by varying the solute concentration at a given temperature. Neutral solutes usually form a straight line (linear isotherm) except for extremely high concentration, indicating the stable partition coefficient (K). However,

when charged analytes are partitioned in a neutral two-phase solvent system, the isotherm tends to form a curved line (non-linear isotherm) due to self-protonation or deprotonation, which alters their K values. If this occurs within a chromatographic column, the solute forms a skewed peak characterized by its prolonged front and steep trailing border.

Partition coefficient (K): the partition coefficient is a ratio of the solute concentration between equilibrated two immiscible solvent phases at a given temperature (usually at around 20 °C). Conventionally K is expressed as the solute concentration in the stationary phase divided by that in the mobile phase as in liquid chromatography. The most suitable K values for HSCCC is $0.5 \leq K \leq 1$, while the best K value for other CCC systems and CCD is around 1. The difference in the suitable K range between HSCCC and other CCC systems derives from an efficient mixing of the two phases in the HSCCC column, which tends to produce longitudinal spreading of the solute band for the sample with large K values. Since one can use either upper or lower phase as the stationary phase in CCC, it is convenient to express the ratio temporarily as solute concentration in the upper phase divided by that in the lower phase, and to avoid a confusion it should be specified as K_{UL} . Thus, if K_{UL} is 2, one should use the lower phase as the stationary phase to make $K=0.5$. If $K_{UL}=1$, either phase can be the stationary phase to get $K=1$. As explained above (in hydrodynamic equilibrium), if one has a choice, the upper phase is more suitable to be used as the stationary phase for various reasons, and therefore it is recommended to bring $0.5 \leq K_{UL} \leq 1$ and use the upper phase as the stationary phase. (Recently, IUPAC recommended that distribution ratio, D , should be used for non-equilibrium situations in HSCCC.)

Partition efficiency: the efficiency of separation in HSCCC may be expressed by two terms, i.e., theoretical plate number (TP) and peak resolution (R_s), each of which is described below. The partition efficiency increases with an increasing the tube length and reducing its i.d.

Peak resolution (R_s): the resolution between two solute peaks is measured using the following equation: $R_s = 2(R_2 - R_1)/(W_2 + W_1)$, where R_1 and R_2 are the retention volumes of the first and the second peaks, and W_1 and W_2 , their peak widths. The separation of $R_s = 1$ gives 95% pure material and $R_s = 1.5$, 99.7% pure material (baseline separation).

pH-zone-refining CCC: pH-zone-refining CCC produces a train of fused rectangular solute peaks called pH-zones, each with its specific pH. Each zone contains highly concentrated solute often near saturation level. It is separated from the neighboring zone by a narrow strip called mixing zone, which contains a mixture of impurities.

Retainer and eluter: these are the most important reagents in pH-zone-refining CCC. The retainer is added to the stationary organic phase to retain the analytes, and the eluter added to the mobile aqueous phase to elute the analytes. TFA and triethylamine are typical retainers for the separation of acidic and basic compounds, respectively, where the stationary phase typically is the organic phase. The retainer determines the analyte concentration in the stationary phase. NH_4OH and HCl are commonly used as the eluter for the separation of acidic and basic compounds, respectively. The eluter always stays in the aqueous mobile phase to serve as a counterion. pH-zone-refining CCC can also be performed using the organic phase as the mobile phase (normal displacement mode). In this case the retainer becomes the eluter to elute the analytes, and the eluter becomes the retainer to retain the analytes in the aqueous stationary phase.

Retention of the stationary phase: a volume of the stationary phase (V_S), which stays permanently within the column during the separation. It is one of the most important parameters to determine the resolution. It is expressed as a percentage relative to the total column capacity (V_C). High retention of 70% is expected from a solvent system with a short settling time of less than 10 s, while the retention of over 50% is satisfactory. It is improved by reducing the flow rate of the mobile phase.

Separation factor: the ratio of partition coefficients between two solutes is called separation factor (α) (in some cases β) (K_1/K_2 , $K_1 \geq K_2$). It is an important parameter, which indicates the peak resolution between the two components. According to our experience, in the commercial HSCCC centrifuge (semipreparative column, 1.6 mm i.d. and ca. 300 ml capacity), $\alpha > 1.5$ is needed to resolve two peaks at the suitable K values and over 50% retention of the stationary phase. For a given pair of two solutes, α varies with the hydrophobicity of the solvent system as well as its composition.

Settling time: this is a time required for the two phases (2 ml each) to settle into two clear layers after gently mixed in a test tube (13 mm \times 100 mm) or graduated cylinder (5 ml capacity). The test may be performed by capping the container, which is gently inverted for five times to thoroughly mix the contents. However, vigorous shaking of the contents for five times showed only a slight difference of the settling time. It has been shown that the settling time well correlates with the retention of the stationary phase in the coiled column in HSCCC. The shorter the settling time, the higher is the retention of the stationary phase. If it is shorter than 10 s, one can expect over 70% retention of the stationary phase. It is desirable that the settling time is less than 20 s, which usually gives over 50% retention under a proper flow rate of the mobile phase.

Stationary phase: conventional liquid chromatography uses a solid support in the column which is called stationary phase. CCC uses no solid support and instead retains one liquid phase of the equilibrated two-phase solvent system in the column as the stationary phase. In HSCCC, the stationary phase is retained in the column utilizing the combined effect of a multilayer coil configuration and a special type of synchronous planetary motion (type-J shown in Fig. 1). For good results the amount of the stationary phase retained in the column should be over 50% of the total column capacity.

Theoretical plate (TP): the TP is computed from the chromatogram according to the conventional formula, i.e., $\text{TP} = (4R/W)^2$, where R is the retention volume (or time) of the peak maximum and W the peak width (the base of the triangle fitted to the peak) measured in volume (or time). In CCC, TP changes with the amount of stationary phase retained in the column, e.g., TP increases as the stationary phase retention decreases approaching infinity at 0% retention. Consequently, TP can be used as a valid parameter only when the retention of the stationary phase is within a reasonable range such as over 50%.

Two-phase solvent system: each system consists of a pair of mutually immiscible solvents, upper and lower phases, usually equilibrated in a separatory funnel. It is divided into three types, organic–organic, organic–aqueous and aqueous–aqueous. Among those the aqueous–aqueous solvent system is free of organic solvent and mainly used for separation of biopolymers such as proteins, but it is not efficiently applied for HSCCC due to extensive emulsification by vigorous mixing in the column. HSCCC successfully uses organic–organic and organic–aqueous solvent systems, which are classified according to the hydrophobicity of the less polar phase. Hydrophobic solvent systems such as hexane–acetonitrile and hexane–ethanol–water are used for separation of non-polar compounds such as fatty acids while hydrophilic solvent system such as butanol–water is used for separation of polar compounds such as peptides. Intermediate solvent systems such as hexane–ethyl acetate–methanol–water and chloroform–methanol–water are useful for separation of moderately hydrophobic compounds. Binary solvent systems such as ethyl acetate–water and butanol–water have a particular feature that the composition of each phase is independent of a volume ratio of the two solvents. The amount of the solvent system required for HSCCC is considerably less than that for preparative HPLC since the analytes are eluted in a more concentrated state especially in pH-zone-refining CCC where the analytes are almost saturated in the mobile phase.

Type-J coil planet centrifuge: a centrifuge, which provides type-J synchronous planetary motion to the column holder the axis of which is parallel to the centrifuge axis (see Fig. 1).

Type-J Synchronous planetary motion: a unique type of planetary motion (so called because the flow tubes form a letter J figure as shown in Fig. 1) of the column holder, which rotates around its own axis once during one revolution around the central axis of the centrifuge in the same direction. The system prevents the flow tubes from twisting during column rotation, and produces an excellent mixing and high retention of the stationary phase in a multilayer coil separation column.

Zone pH (pH_{zone}): it indicates pH of the solute zone in pH-zone-refining CCC which is expressed by $pH_{zone} = pK_a + \log[(K_{DS}/K_S) - 1]$, where K_{DS} is the partition ratio of solute S (an indicator for solute hydrophobicity) and K_S , the dissociation constant of the solute. According to the theoretical analysis two solutes, which differ their zone pH values by 0.2 or greater can be resolved in pH-zone-refining CCC.